REMARKS

Claims 1-18 and 20-48 were pending in this application. Claims 1-14 and 33-47 have been withdrawn from consideration as being directed to non-elected inventions. Claim 27 has been amended to clarify its content.

Support for amended claim 27 can be found on page 35, lines 7-24 and on page 62, lines 7-11 of the instant application. Thus, as a result of the foregoing amendment, claims 15-18, 20-32 and 48 remain for consideration.

No new matter has been added by way of this amendment. Reconsideration of this application is respectfully requested.

Claim Rejections under 35 U.S.C. §112, second paragraph

Claims 15-32 are rejected under 35 U.S.C. §112, second paragraph, as being indefinite. The Examiner alleges that claim 27 recites the limitation "wherein the two homologous nucleotide sequences are IRESEGFP" and that the exact nucleotide sequence encompassed by this term is not described in the instant application and does not appear to be a consensus sequence known in the art.

Applicants respectfully traverse this rejection and have amended claim 27 to clarify the usage of the term "IRESEGFP". Furthermore, Applicants provide for the Examiner's convenience a copy of two US Patents, Nos. 5,491,084 and 5,777,079 drawn to green fluorescent proteins and their uses. Applicants respectfully refer the Examiner to the filing date of both issued patents to clearly point out to the Examiner that such marker proteins, and the nucleic acid sequences encoding such proteins were known to those skilled in the art at the time the present application was filed. Applicants believe that the claim amendment as well as the support for the level of skill in the art at the time the instant application was filed would thus obviate the rejection of this claim as well as any dependent claims. In light of the foregoing, Applicants respectfully request withdrawal of the rejection.

Double Patenting

Claims 15-18, 20, 25-26, 29, and 48 have been rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1, and 6-7 of U.S. Patent No. 6,485,912. Applicants respectfully traverse the Examiner's rejection and herewith file a Terminal Disclaimer in compliance with 37 CFR 1.321(c), disclaiming the term of any patent granted on the instant application beyond that of the aforementioned patent. In light of the foregoing, withdrawal of the rejection is respectfully requested.

Fees

No fees are believed to be required, but if so, the Commissioner is hereby authorized to charge any fees, or credit any overpayment, to Deposit Account No. 11-1153.

Conclusion

Applicants believe that the foregoing amendments to the claims and the submission of a Terminal Disclaimer place the application in condition for allowance. Withdrawal of the rejections is respectfully requested. If a discussion with the undersigned will be of assistance in resolving any remaining issues, the Examiner is invited to telephone the undersigned at (201) 487-5800, ext. 118, to effect a resolution.

Respectfully submitted,

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Date: July 9, 2003

Enclosures: Terminal Disclaimer
US Patent Nos: 5,491,084 and 5,777,079

09/619,364
#20
Exhibit B

US005491084A

United States Patent [19]

Chalfie et al.

[11] Patent Number: 5,491,084

[45] Date of Patent: Feb. 13, 1996

[54] USES OF GREEN-FLUORESCENT PROTEIN

[75] Inventors: **Martin Chalfie**, New York, N.Y.; **Douglas Prasher**, East Falmouth, Mass.

[73] Assignees: **The Trustees of Columbia University in the City of New York**, New York, N.Y.; **Woods Hole Oceanographic Institution**, Woods Hole, Mass.

[21] Appl. No.: **119,678**

[22] Filed: **Sep. 10, 1993**

[51] **Int. Cl.**[6] **C12N 9/02**; C12N 5/00; C12P 21/06; C07H 19/00

[52] **U.S. Cl.** **435/189**; 435/69.1; 435/69.7; 435/240.2; 435/252.3; 435/320.1; 536/22.1; 536/23.1; 536/23.4; 536/23.5

[58] **Field of Search** 435/69.1, 69.7, 435/189, 240.2, 252.3, 320.1; 536/22.1, 23.1, 23.4, 23.5

[56] **References Cited**

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Gould, S. J., and subrami, S., *Anal. Biochem.*, 175:5 (1988) (Exhibit D).

Silhavy, T. J., and Beckwith, J. R., *Microbiol. Rev.*, 49:398 (1985) (Exhibit E).

Stewart, G. S. A. B., and Williams, P., *J. Gen. Microbiol.*, 138:1289 (1992) (Exhibit F).

Prasher et al. "Primary structure of the *Dequorea victoria* . . . " Gene 111 pp. 229–233 1992.

Glover "Expression of cloned genes in animal cells" *Gene cloning* pp. 179–213 1984.

Primary Examiner—Robert A. Wax
Assistant Examiner—Hyosuk Kim
Attorney, Agent, or Firm—John P. White

[57] **ABSTRACT**

This invention provides a cell comprising a DNA molecule having a regulatory element from a gene, other than a gene encoding a green-fluorescent protein operatively linked to a DNA sequence encoding the green-fluorescent protein. This invention also provides a method for selecting cells expressing a protein of interest which comprises: a. introducing into the cells a DNAI molecule having DNA sequence encoding the protein of interest and DNAII molecule having DNA sequence encoding a green-fluorescent protein; b. culturing the introduced cells in conditions permitting expression of the green-fluorescent protein and the protein of interest; and c. selecting the cultured cells which express green-fluorescent protein, thereby selecting cells expressing the protein of interest. Finally, this invention provides various uses of a green-fluorescent protein.

12 Claims, 3 Drawing Sheets

FIGURE 1

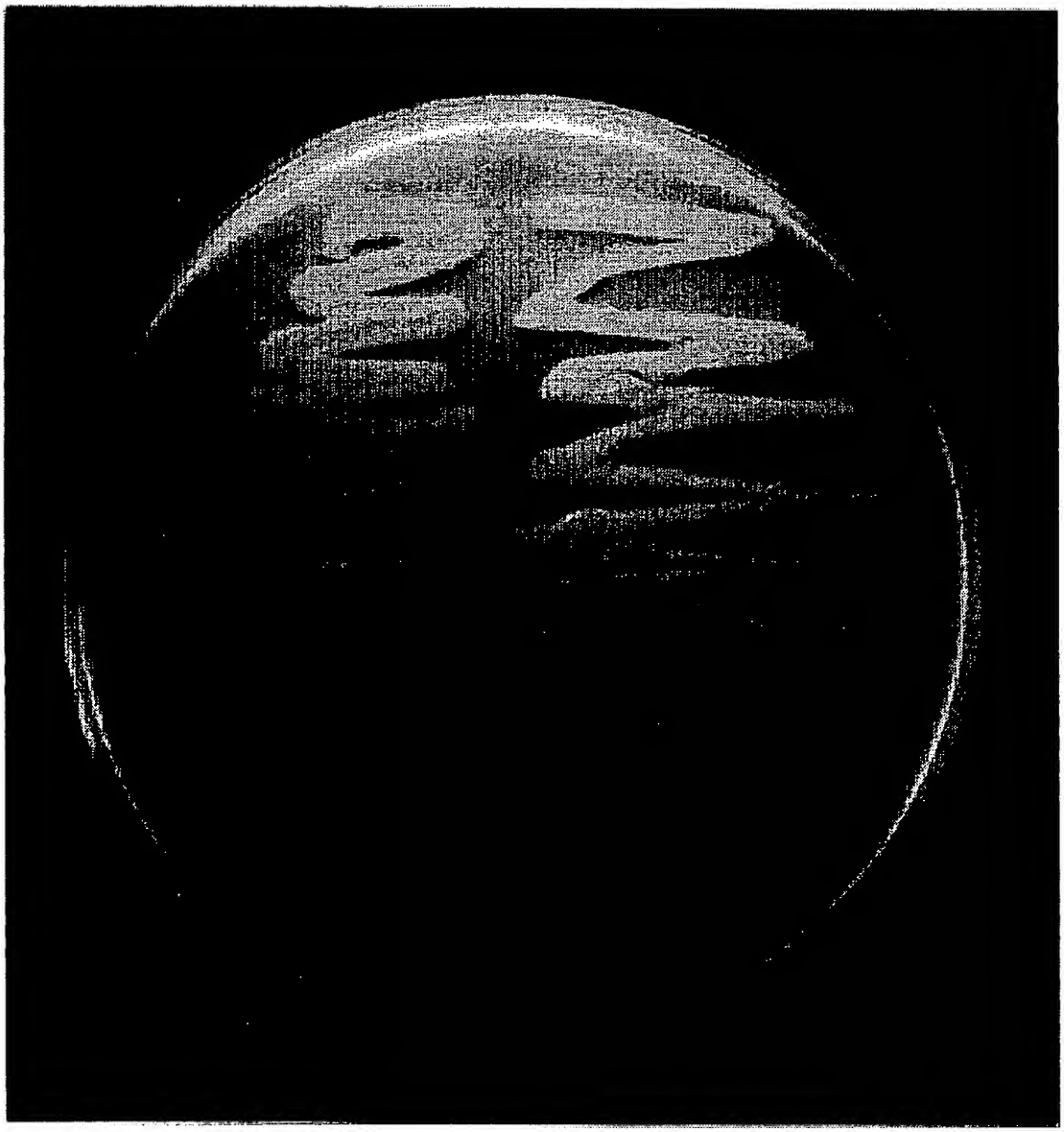
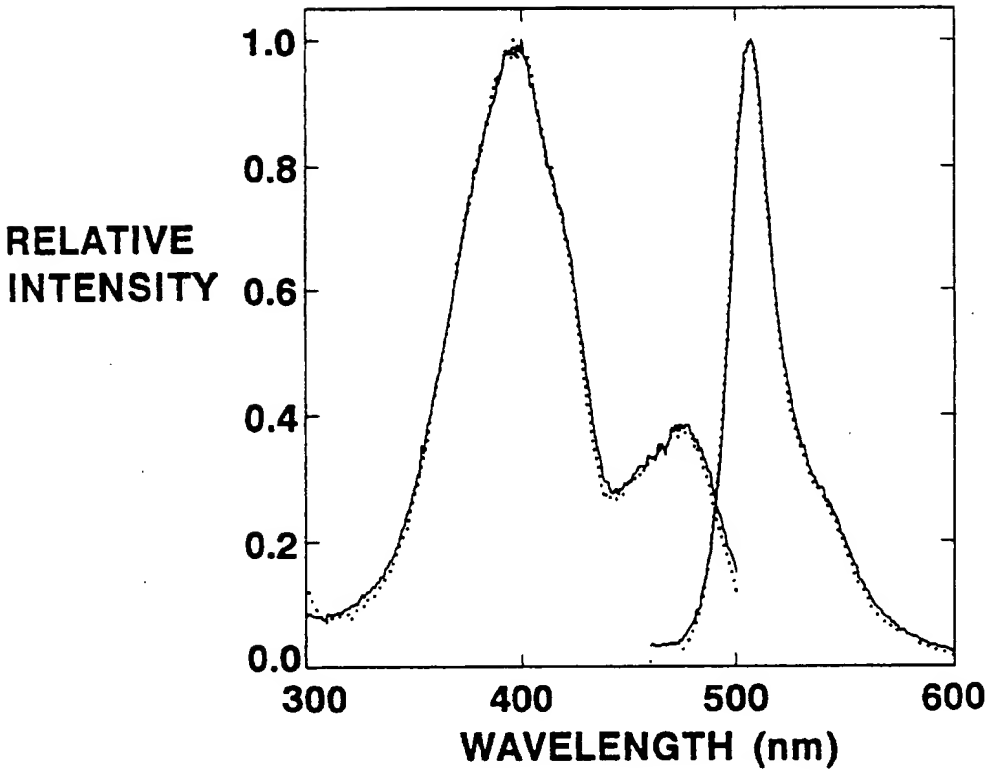


FIGURE 2


RELATIVE INTENSITY
1.0
0.8
0.6
0.4
0.2
0.0
300
400
500
600
WAVELENGTH (nm)

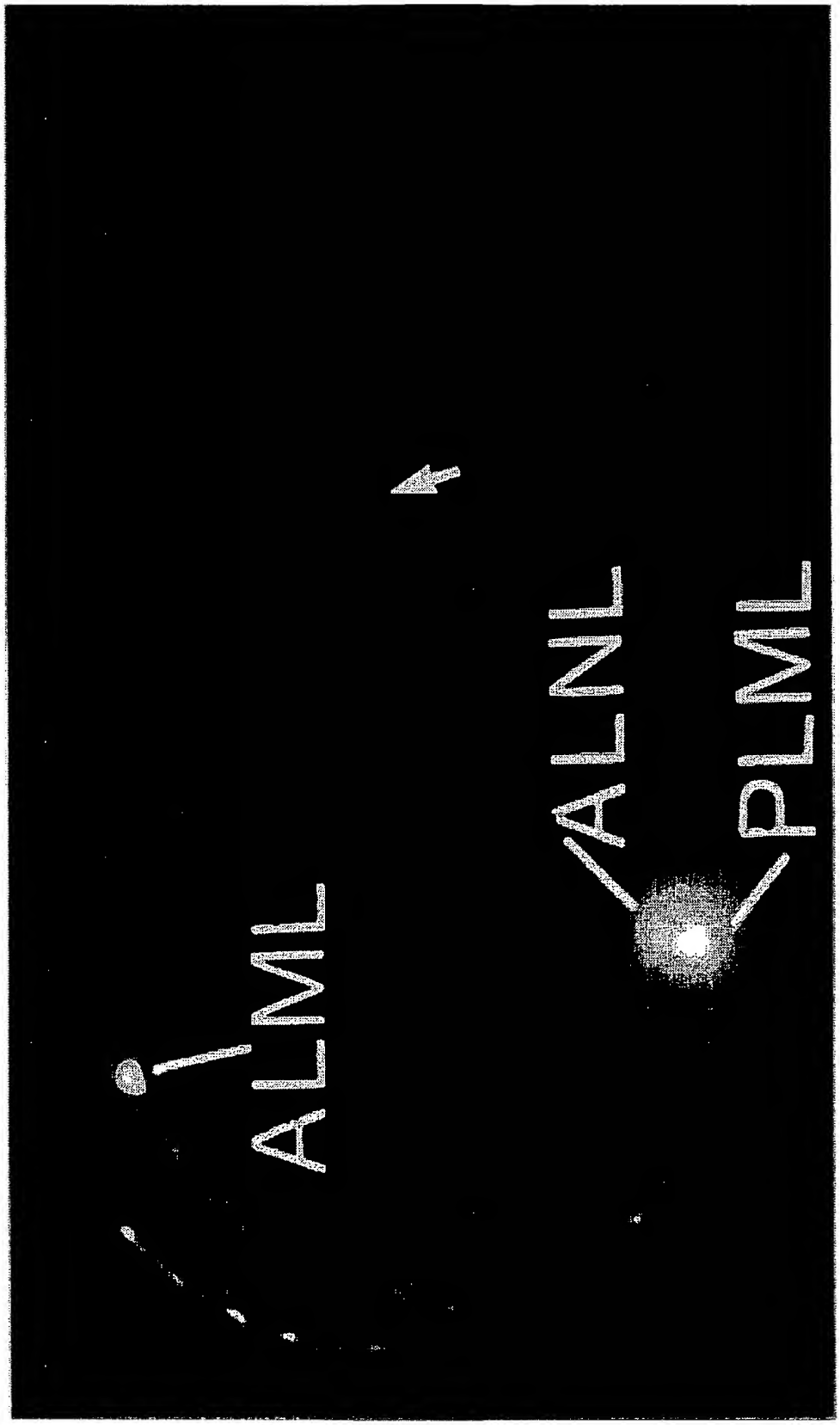


FIGURE 3

USES OF GREEN-FLUORESCENT PROTEIN

The invention disclosed herein was made with Government support under NIH Grant No. 5R01GM30997 from the Department of Health and Human Services. Accordingly, the U.S. Government has certain rights in this invention.

BACKGROUND OF THE INVENTION

Throughout this application various references are referred to within parenthesis. Disclosures of these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains. Full bibliographic citation for these references may be found at the end of this application, preceding the sequence listing and the claims.

Several methods are available to monitor gene activity and protein distribution within cells. These include the formation of fusion proteins with coding sequences for β-galactosidase (21), and luciferases (21). The usefulness of these methods is often limited by the requirement to fix cell preparations or to add exogenous substrates or cofactors. This invention disclose a method of examining gene expression and protein localization in living cells that requires no exogenously-added compounds.

This method uses a cDNA encoding the Green-Fluorescent Protein (GFP) from the jelly fish *Aequorea victoria* (5). In A. victoria, GFP absorbs light generated by aequorin upon the addition of calcium and emits a green light.

This invention discloses that GFP expressed in prokaryotic and eukaryotic cells is capable of producing a strong green fluorescence when excited near UV or blue light. Since this fluorescence requires no additional gene products from *A. victoria*, chromophore formation is not species specific.

SUMMARY OF THE INVENTION

This invention provides a cell comprising a DNA molecule having a regulatory element from a gene, other than a gene encoding a green-fluorescent protein operatively linked to a DNA sequence encoding the green-fluorescent protein.

This invention provides a method for selecting cells expressing a protein of interest which comprises: a) introducing into the cells a DNAI molecule having DNA sequence encoding the protein of interest and DNAII molecule having DNA sequence encoding a green-fluorescent protein; b) culturing the introduced cells in conditions permitting expression of the green-fluorescent protein and the protein of interest; and c) selecting the cultured cells which express green-fluorescent protein, thereby selecting cells expressing the protein of interest.

This invention also provides a method for localizing a protein of interest in a cell: a. introducing into a cell a DNA molecule having DNA sequence encoding the protein of interest linked to DNA sequence encoding a green-fluorescent protein such that the protein produced by the DNA molecule will have the protein of interest fused to the green-fluorescent protein; b. culturing the cell in conditions permitting expression of the fused protein; c. detecting the location of the green-fluorescent protein in the cell, thereby localizing a protein of interest in a cell.

BRIEF DESCRIPTION OF FIGURES

FIG. 1 Expression of GFP in *E. coli*. The bacteria on the right side of the figure have the GFP expression plasmid. This photograph was taken while irradiating the plate with a hand-held long-wave UV source.

FIG. 2 Excitation and Emission Spectra of *E. coli*-generated GFP (solid lines) and purified *A. victoria* GFP (L form; dotted lines).

FIG. 3 Expression of GFP in a first stage Caenorhabditis elegans larva. Two touch receptor neurons (PLML and ALML) and one other neuron of unknown function (ALNL) are indicated. Processes can be seen projecting from all three cell bodies. The arrow points to the nerve ring branch from the ALML cell (out of focus). The background fluorescence is due to the animal's autofluorescence.

DETAILED DESCRIPTION OF THE INVENTION

Throughout this application, the following standard abbreviations are used to indicate specific nucleotides:

C = cytosine	A = adenosine
T = thymidine	G = guanosine

This invention provides a cell comprising a DNA molecule having a regulatory element from a gene, other than a gene encoding a green-fluorescent protein operatively linked to a DNA sequence encoding the green-fluorescent protein.

This invention provides a cell comprising a DNA molecule having a regulatory element from a gene, other than a gene encoding a green-fluorescent protein operatively linked to a DNA sequence encoding the green-fluorescent protein, wherein the cell is selected from a group consisting essentially of bacterial cell, yeast cell, fungal cell, insect cell, nematode cell, plant or animal cell.

Suitable animal cells include, but are not limited to Vero cells, HeLa cells, Cos cells, CV1 cells and various primary mammalian cells.

In an embodiment, the bacterial cell is *Escherichia coli*.

As used herein, "a regulatory element" from a gene is the DNA sequence which is necessary for the expression of the gene.

In this invention, the term "operatively linked" to means that following such a link the regulatory element can direct the expression of the linked DNA sequence which encodes a green-fluorescent protein.

The gene encoding a green-fluorescent protein includes DNA molecules coding for polypeptide analogs, fragments or derivatives of antigenic polypeptides which differ from naturally-occurring forms in terms of the identity or location of one or more amino acid residues (deletion analogs containing less than all of the residues specified for the protein, substitution analogs wherein one or more residues specified are replaced by other residues and addition analogs where in one or more amino acid residues is added to a terminal or medial portion of the polypeptides) and which share some or all properties of naturally-occurring forms.

These DNA molecules include: the incorporation of codons "preferred" for expression by selected non-mammalian hosts; the provision of sites for cleavage by restriction endonuclease enzymes; and the provision of additional initial, terminal or intermediate DNA sequences that facilitate construction of readily expressed vectors.

As an example, plasmid pGFP10.1 codes for a mutated GFP protein with the 80th codon changed from a glutamine to arginine. This mutated protein retains the property of the natural protein.

In an embodiment, the regulatory element is a promoter. In a further embodiment, the promoter is activated by a heavy metal. Such promoters are well-known in the art (J. H.

Freedman, L. W. Slice, A. Fire, and C. S. Rubin (1993) *Journal of Biological Chemistry,* 268:2554).

In another embodiment, the promoter is a P450 promoter. Cytochrome P450 is well-known in the art and there are a number of P450 promoters known.

In still another embodiment, the promoter is for a stress protein. Such stress proteins are well-known in the art (E. G. Stringham, D. K. Dixon, D. Jones and E. D. Candido (1992) *Molecular Biology of the Cell,* 3:221; and William J. Welch (May, 1993), *Scientific American,* page 56). In a further embodiment, the stress protein is a heat-shock protein.

This invention provides a cell comprising a DNA molecule having a regulatory element from a gene, other than a gene encoding a green-fluorescent protein operatively linked to a DNA sequence encoding the green-fluorescent protein, wherein the promoter is for a gene viable to the cell growth.

In another embodiment, the regulatory element is an enhancer. Enhancers are well-known in the art.

This invention provides a cell comprising a DNA molecule having a regulatory element from a gene, other than a gene encoding a green-fluorescent protein operatively linked to a DNA sequence encoding the green-fluorescent protein, wherein the DNA sequence encoding the *Aequorea victoria* green-fluorescent protein.

In an embodiment, the *Aequorea victoria* green-fluorescent protein is cloned in a plasmid. This plasmid is a modification of the pBS(+) (formerly called pBluescribe +) vector (Stratagene®) which has inserted within it an Eco RI fragment containing the cDNA sequence of the *Aequorea victoria* green-fluorescent protein (as modified herein). The fragment was obtained from λGFP10 (Prasher, D. C., Eckenrode, V. K., Ward, W. W., Prendergast, F. G., and Cormier, M. J., (1992) Primary structure of the *Aequorea victoria* green-fluorescent protein. *Gene,* 111:229–233) by amplification using the polymerase chain reaction (Saiki, R. K., Gelfand, D. H., Stoffel, S., Sharf, S. J., Higuchi, G. T., Horn, G. T., Mullis, K. B., and Erlich, H. A. (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science,* 239:487–491) with primers flanking the Eco RI sites and subsequent digestion with Eco RI. The sequence of the cDNA in pGFP10.1 differs from the published sequence (5) by a change of the 80th codon of the coding sequence from CAG to CGG, a change that produces a glutamine to arginine change in the protein sequence.

This pGFP10.1 plasmid was deposited on Sep. 1, 1993 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Md. 20852, U.S.A. under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganism for the Purposes of Patent Procedure. Plasmid pGFP10.1 was accorded ATCC Accession Number 75547.

This invention provides a living organism comprising the cell comprising a DNA molecule having a regulatory element from a gene, other than a gene encoding a green-fluorescent protein operatively linked to a DNA sequence encoding the green-fluorescent protein.

In an embodiment, the living organism is *C. elegans.* In another embodiment, the living organism is Drosophila, zebra fish or bacteriophage.

A bacteriophage carrying the green-fluorescent protein gene can infect a particular type of bacteria. The infection may be easily detected via the expression of the green-fluorescent protein. Therefore, by using appropriate bacteriophages, the presence of that particular type of bacteria may be detected.

Similarly, a virus carrying the green-fluorescent protein gene may infect a specific cell type. The infection may be easily detected by monitoring the expression of the green-fluorescent protein.

The above-described cells and living organisms are useful to detect effects of external stimulus to the regulatory element. The stimulus may have direct or indirect effects on the regulatory element. Such effects will be detectable through either the induction of expression and production of the green-fluorescent protein or switching off the expression of the green-fluorescent protein.

Cells expressing the green-fluorescent proteins may be conveniently separated by a fluorescence-activated cell sorter.

These cells and organisms may be used to detect the presence of different molecules in various kinds of biological samples such as blood, urine or saliva. By operatively linking a regulatory element of the gene which is affected by the molecule of interest to a green-fluorescent protein, the presence of the molecules will affect the regulatory element which in turn will affect the expression of the green-fluorescent protein. Therefore, the above-described cells are useful for the detection of molecules. Such detection may be used for diagnostic purposes. An example of such a molecule is a hormone.

This invention provides a living organism comprising the cell comprising a DNA molecule having a regulatory element from a gene, other than a gene encoding a green-fluorescent protein operatively linked to a DNA sequence encoding the green-fluorescent protein, wherein the regulatory element is for a stress protein.

This invention provides a living organism comprising the cell comprising a DNA molecule having a regulatory element from a gene, other than a gene encoding a green-fluorescent protein operatively linked to a DNA sequence encoding the green-fluorescent protein, wherein the stress protein is a heat-shock protein.

This invention provides a method for selecting cells expressing a protein of interest which comprises: a) introducing into the cells a DNAI molecule having DNA sequence encoding the protein of interest and DNAII molecule having DNA sequence encoding a green-fluorescent protein; b) culturing the introduced cells in conditions permitting expression of the green-fluorescent protein and the protein of interest; and c) selecting the cultured cells which express green-fluorescent protein, thereby selecting cells expressing the protein of interest.

This invention also provides the above method, wherein the cells are selected from a group consisting essentially of bacterial cells, yeast cells, fungal cells, insect cells, nematode cells, plant or animal cells. Suitable animal cells include, but are not limited to Vero cells, HeLa cells, Cos cells, CV1 cells and various primary mammalian cells.

In an embodiment, DNAI and DNAII are linked. In another embodiment, the DNA encodes the *Aequorea victoria* green-fluorescent protein.

This invention provides a method for localizing a protein of interest in a cell which comprises: a) introducing into a cell a DNA molecule having DNA sequence encoding the protein of interest linked to DNA sequence encoding a green-fluorescent protein such that the protein produced by the DNA molecule will have the protein of interest fused to the green-fluorescent protein; b) culturing the cell in conditions permitting expression of the fused protein; and c) detecting the location of the green-fluorescent protein in the cell, thereby localizing a protein of interest in a cell.

Regulatory elements required for expression include promoter sequences to bind RNA polymerase and transcription initiation sequences for ribosome binding. For example, a

bacterial expression vector includes a promoter such as the lac promoter and for transcription initiation the Shine-Dalgarno sequence and the start codon AUG. Similarly, a eukaryotic expression vector includes a heterologous or homologous promoter for RNA polymerase II, a downstream polyadenylation signal, the start codon AUG, and a termination codon for detachment of the ribosome. Such vectors may be obtained commercially or assembled from the sequences described by methods well-known in the art, for example the methods described above for constructing vectors in general.

The host cell of the above expression system may be selected from the group consisting of the cells where the protein of interest is normally expressed, or foreign cells such as bacterial cells (such as *E. coli*), yeast cells, fungal cells, insect cells, nematode cells, plant or animal cells, where the protein of interest is not normally expressed. Suitable animal cells include, but are not limited to Veto cells, HeLa cells, Cos cells, CV1 cells and various primary mammalian cells.

In an embodiment of the method for localizing a protein of interest in a cell, the DNA encoding the green-fluorescent protein is from *Aequorea victoria.*

This invention provides a method for localizing a protein of interest in a cell which comprises: a) introducing into a cell a DNA molecule having DNA sequence encoding the protein of interest linked to DNA sequence encoding a green-fluorescent protein such that the protein produced by the DNA molecule will have the protein of interest fused to the green-fluorescent protein; b) culturing the cell in conditions permitting expression of the fused protein; and c) detecting the location of the green-fluorescent protein in the cell, thereby localizing a protein of interest in a cell, wherein the cell normally expressing the protein of interest.

This invention provides a method for detecting expression of a gene in a cell which comprises: a) introducing into the cell a DNA molecule having DNA sequence of the gene linked to DNA sequence encoding a green-fluorescent protein such that the regulatory element of the gene will control expression of the green-fluorescent protein; b) culturing the cell in conditions permitting expression of the gene; and c) detecting the expression of the green-fluorescent protein in the cell, thereby indicating the expression of the green in the cell.

This invention provides a method for indicating expression of a gene in a subject which comprises: a) introducing into a cell of the subject a DNA molecule having DNA sequence of the gene linked to DNA sequence encoding a green-fluorescent protein such that the regulatory element of the gene will control expression of the green-fluorescent protein; b) culturing the cell in conditions permitting expression of the fused protein; and c) detecting the expression of the green-fluorescent protein in the cell, thereby indicating the expression of the gene in the cell.

In an embodiment of the above methods, the green-fluorescent protein is the *Aequorea victoria* green-fluorescent protein.

This invention provides a method for determining the tissue-specificity of a DNA sequence in a subject which comprises: a) introducing into a cell of the subject a DNA molecule having the DNA sequence linked to DNA sequence encoding a green-fluorescent protein such that the DNA sequence will control expression of the green-fluorescent protein; b) culturing the subject in conditions permitting the expression of the green-fluorescent protein; and c) detecting the expression of the green-fluorescent protein in different tissue of the subject, thereby determining the tissue-specificity of the DNA sequence.

This invention provides a method for determining the presence of heavy metal in a solution which comprises: a) culturing the cell comprising a DNA molecule having a promoter from a gene, other than a green-fluorescent protein operatively linked to a DNA sequence encoding the green-fluorescent protein, wherein the promoter is activated by a heavy metal in the solution; and b) detecting expression of the green-fluorescent protein, the expression of the green-fluorescent protein indicates the presence of heavy metal.

This invention provides a method for detecting pollutants in a solution which comprises: a) culturing the cell comprising a DNA molecule having a promoter from a gene, other than a green-fluorescent protein operatively linked to a DNA sequence encoding the green-fluorescent protein, wherein the promoter is activated by a heavy metal or the promoter is for a stress protein in the solution; and b) detecting expression of the green-fluorescent protein, the expression of the green-fluorescent protein indicates the presence of heavy metal.

This invention will be better understood from the Experimental Details which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims which follow thereafter.

EXPERIMENTAL DETAILS

Light is produced by the bioluminescent jellyfish *Aequorea victoria* when calcium binds to the photoprotein aequorin (1). Although activation of aequorin in vitro or in heterologous cells produces blue light, the jellyfish produces green light. This latter light is due to the presence of a second protein in *A. victoria* called the green-fluorescent protein (GFP) that absorbs the energy from aequorin (2).

Purified GFP absorbs blue light (maximally at 395 nm with a minor peak at 470 m) and emits green light (peak emission at 509 nm with a shoulder at 540 nm) (2, 3). This fluorescence is very stable; virtually no photobleaching is observed (4). As recently deduced from a cDNA clone from *A. victoria*, GFP is a protein of 238 amino acids (5). Although the intact protein is needed for fluorescence, the same absorption spectral properties as the denatured protein are found in a hexapeptide starting at amino acid 64 (6, 7). The GFP chromophore is derived from the primary amino acid sequence through the cyclization of Ser-dehydroTyr-Gly within this hexapeptide (7). The mechanisms that produce the dehydrotyrosine and cyclize the polypeptide to form the chromophore are unknown. To determine whether additional factors from *A. victoria* were needed for the production of the fluorescent protein, applicants tested GFP fluorescence in heterologous systems. Applicants show here that GFP expressed in prokaryotic and eukaryotic cells is capable of producing a strong green fluorescence when excited by blue light. Since this fluorescence requires no additional gene products from *A. victoria*, chromophore formation is not species specific and occurs either through the uses of ubiquitous cellular components or by autocatalysis.

Expression of GFP in *Escherichia coli* (8) results in an easily detected green fluorescence (9) that is not seen in control bacteria. For example, florescent bacteria were easily seen on plates containing IPTG when they were illuminated with a long-wave UV source (FIG. 1). Since the cells grow well in the continual presence of the inducer (IPTG), GFP does not appear to have a toxic effect on the cells. When GFP was partially purified from this strain (10), it was found to have fluorescence excitation and emission spectra indistin-

guishable form those of the purified native protein (FIG. 2). The spectral properties of the recombinant GFP suggest that the chromophore can form in the absence of other *A. victoria* products.

Transformation of the nematode *Caenorhabditis elegans* also resulted in the production of fluorescent GFP (11) (FIG. 3). GFP expression was directed to a relatively small number of neurons using a promoter for the mec-7 gene. This gene encodes a β-tubulin (12) that is expressed strongly in six touch receptor neurons in *C. elegans* and weakly in a few other neurons (13, 14). The pattern of expression of GFP was similar to that seen with the anti-MEC-7 antibody or from mec-71acZ fusions (13–15). The strongest fluorescence was seen in the cell bodies of the four embryonically-derived touch receptor neurons (ALML, ALMR, PLML, PLMR). The processes from these cells, including their terminal branches, were often easily seen in larval animals. In older larvae, the cell bodies of the remaining touch cells (AVM and PVM) were also seen; the processes of these cells were more difficult to detect. These postembryonically-derived cells arise during the first of the four larval stages (16), but their outgrowth occurs in the following larval stages (17) with the cells becoming functional during the fourth larval stage (18). GFP's fluorescence in these cells is consistent with these previous results; no newly hatched or late first-stage larvae, and seven of eight young adults had at least one of these cells (19). In addition, moderate to weak fluorescence was seen for a few other neurons (FIGS. 3, and 20).

The expression of GFP in both *E. coli* and *C. elegans* differs from that of the native protein in one respect. Although relatively stable when illuminated using low intensities of 470 nm light, the fluorescence photobleaches rapidly (within seconds) when the cells are illuminated with light of approximately 395 nm. Applicants do not know whether this difference is caused by the alteration of the protein-coding sequence (8), by the absence of a necessary post-translational modification, or by non-specific damage within the cells. However, when cells in *C. elegans* have been photobleached, some recovery is seen within 10 minutes. Further investigation is needed to determine whether this recovery represents de novo synthesis of GFP.

Several methods are available to monitor gene activity and protein distribution within cells. These include the formation of fusion proteins with coding sequences for β-galactosidase, firefly luciferase, and bacterial luciferase (21). Because these methods require exogenously-added substrates or cofactors, they are of limited use with living tissue. Since detection of intracellular GFP requires only the radiation by near UV or blue light, it is not substrate limited. Thus, it should provide an excellent means of monitoring gene expression and protein localization in living cells (22).

Because it does not appear to interfere with cell growth and function, GFP should also be a convenient indicator of transformation (and one that could allow cells to be separated using fluorescence-activated cell sorting). Applicants also envision that GFP can be used as a vital marker so that cell growth (for example, the elaboration of neuronal processes) and movement can be followed in situ, especially in animals that are essentially transparent like *C. elegans* and zebra fish.

References and notes

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5. Prasher, D. C., Eckenrode, V. K., Ward, W. W., Prendergast, F. G., and Cormier, M. J., (1992), *Gene*, 111:229.

6. Shimomura, A., (1979), *FEBS Lett.*, 104:220.

7. Cody, C. W., Prasher, D. C., Westler, W. M., Prendergast, F. G., and Ward, W. W., (1993), *Biochemistry*, 32:1212.

8. Plasmid pGFP10.1 was formed by placing the Eco RI fragment encoding the GFP cDNA from λgfp10 (5) into pBS(+) (Stratagene®). The fragment was obtained by amplification using the polymerase chain reaction (PCR; R. K. Saiki, et al., *Science*, 239:487 (1988)) with primers flanking the Eco RI sites and subsequent digestion with Eco RI. The sequence of the cDNA in pGFP10.1 differs from the published sequence by a change in codon 80 within the coding sequence from CAG to CGG, a change that replaces a glutamine residue with arginine. As seen in FIG. 2, this replacement has no detectable effect on the spectral properties of the protein.

An *E. coli* expression construct was made by using PCR to generate a fragment with an Nbe I site at the start of translation and an Eco RI site 5' primer was ACAAAGGCTAGCAAAGGAGAAGAAC (Sequence ID No.: 1) and the 3' primer was the T3 primer (Stratagene®). The Nhe I-Eco RI fragment was ligated into the similarly cut vector pET3a [A. H. Rosenberg., et al., *Gene*, 56:125, (1987)] by standard methods [J. Sambrook, E. F., Fritsch, and T. Maniatis, *Molecular cloning: A laboratory manual, 2nd ed.*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., (1989)]. The *E. coli* strain BL21(DE3)Lys [F. W. Studier and B. A. Moffat, *J. Mol. Biol.*, 189:113 (1986)] was transformed with the resulting plasmid (TU#58) and grown at 37°. Control bacteria were transformed with pET3a. Animals were grown on nutrient plates containing 100 μg/ml ampicillin and 0.8 mM IPTG.

9. A variety of microscopes have been used for these experiments with epifluorescence filter sets normally used for fluorescein isothiocyanate. In addition, as might be expected from its spectral characteristics a Xenon lamp gave a more intense fluorescence than a mercury lamp when cells were illuminated with light round 470 nm. No other attempts were made to enhance the signal (for example, by using low intensity light cameras), although it is may be useful in some instances.

10. GFP was purified from 250 ml cultures of BL21(DE3)Lys S bacteria containing TU#58; bacteria were grown in LB broth [J. Sambrook, et al., op. cit.] containing 100 μg/ml ampicillin and 0.8 mM IPTG. Applicants fount that induction was best when IPTG was present continually. Cells were washed in 4 ml of 10 mM Tris. HCl pH 7.4, 100 mM NaCl, 1 mM $MgCl_2$, and 10 mM dithiothreitol [A. Kumagai and W. G. Dunphy, *Cell*, 64:903 (1991)] and then sonicated (2×20 sec) in 4 ml of the same buffer containing 0.1 mM PMSF, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, and 2 μg/ml aprotinin, and centrifuge at 5,000 rpm for 5' in the cold. The supernatant was centrifuged a second time (15,000 rpm for 15') and then diluted seven fold with 10 mM Tris pH 8.0, 10 mM EDTA and 0.02% NAN_3. Corrected excitation and emission spectra were obtained using a SPEX FIT11 Spectrofluormeter and compared with purified L-form GFP from *A. victoria* (M. Cutler, A. Roth and W. W. Ward, unpublished data). The excitation spectra were measured from 300–500 nm with a fixed emission wavelength of 509 nm and the emission spectra, were measured from 410–600 nm with a fixed excitation of 395 nm. All spectra were

recorded as signal/reference data (where the reference is a direct measurement of the lamp intensity using a separate photomultiplier tube) at room temperature with 1 second integration time and 1 nm increment. The spectral hand width were adjusted to 0.94 nm for all spectra.

11. Wild-type and mutant animals were grown and genetic strains were constructed according to S. Brenner, *Genetics*, 77:71 (1974).

The plasmid p3FP10.1 was used as a template in PCR to generate a fragment with a 5' Nhe I site (at the start of translation) and a 3' Eco RI site (3' of the termination codon). The DNA was cut to produce an Nhe I-Eco RI fragment that was ligated into plasmid pPD 16.51 [12; A. Fire, S. W. Harrison, and D. Dixon, *Gene*, 93:189 (1990)], a vector containing the promoter of the *C. elegans* mec-7 gene. Wild-type *C. elegans* were transformed by coinjecting this DNA (TU#64) and the DNA for plasmid pRF4, which contains the dominant rol-6 (su1006) mutation, into adult gonads as described by C. M. Mello, J. M. Kramer, D. Stinchcomb, and V. Ambros, *EMBO J.*, 10:3959 (1991). A relatively stable line was isolated (TU1710) and the DNA it carried was integrated as described by Mitani et al. (15) to produce the integrated elements uIs3 and uIs4 (in strains TU1754 and TU1755, respectively).

Live animals were mounted on agar (or agarose) pads as described by Sulston and Horvitz (16), often with 10 mM NaN_3 as an anesthetic (G. Nelson, pers. comm.) and examined using either a Zeiss universal or axiophot microscope. For *C. elegans*, applicants find that a long-pass emission filter works best, because the animal's intestinal autofluorescence, which increases as the animal matures, appears yellow (with band-pass filters the autofluorescence appears green and obscures the GFP fluorescence).

Because much more intense fluorescence was seen in uIs4 than uIs3 animals (for example, it was often difficult to see the processes of the ALM and PLM cells in uIs3 animals using a mercury lamp), the former have been used entirely for the observations reported here. The general pattern of cell body fluorescence was the same in both strains and in the parental, nonintegrated strain (fluorescence in this strain was strong like in the uIs4 animals). The uIs4 animals, however, did show an unusual phenotype; both the ALM and PLM touch cells were often displaced anteriorly. These cells usually showed the correct pattern of outgrowth, although occasional cells had abnormally projecting processes. These cells could be identified as touch receptor cells, since the fluorescence was dependent on mec-3, a homeobox gene that specifies touch cell fate [13, 15, 18, J. C. Way and M. Chalfie, *Cell*, 54:5 (1988)]. It has been shown previously that mec-7 expression is reduced in the ALM touch cells of the head (but not as dramatically in the PLM touch cells of the tail) in mec-3 gene mutants (13, 15). Applicants find a similar change of GfP expression in a mec-3 mutant background for both uIs3 and uIs4. Thus, GFP accurately mimics the known expression pattern of the mec-7 gene. It is likely that the reduced staining in uIs3 animals and the misplaced cells in uIs4 animals is due either to secondary mutations or the amount and position of the integrated DNA.

12. Savage, C., Hamelin, M., Culotti, J. G., Coulson, A., Albertson, D. G., and Chalfie, M., *Genes. Dev.*, 3:870, (1989).

13. Hamelin, M., Scott, I. M., Way, J. C., Culotti, J. G., (1992) *EMBO J.*, 11:2885.

14. Duggan, A., and Chalfie, M., unpublished data.

15. Mitani, S., Du, H. P., Hall, D. H., Driscoll, M., and Chalfie, M., (1993), *Development*, in press.

16. Sulston, J. E., and Horvitz, H. R., (1977), *Develop. Biol.*, 56:110.

17. Walthall, W. W. and Chalfie, M., (1988), *Science*, 239:643.

18. Chalfie, M., and Sulston, J., (1981), *Dev. Biol.*, 82:358.

19. In adults, the thicker size of the animals and the more intense autofluorescence of the intestine tend to obscure these cells.

20. These include several cells in the head (including the FLP cells) and tail of newly hatched animals and the BDU cells, a pair of neurons just posterior to the pharynx. Such cells have been seen previously (13, 15). The strongest staining of these non-touch receptor neurons are a pair of cells in the tail that have anteriorly-directed processes that project along the dorsal muscle line. It is likely that these are the ALN cells, the sister cells to the PLM touch cells [J. G. White, E. Southgate, J. N. Thomson, and S. Brenner, *Philos. Trans. R. Soc. Lond. B. Biol. Sci*, 314:1 (1986)].

21. Reviewed in T. J. Silhavy and J. R. Beckwith, *Microbiol. Rev.* 49:398 (1985); S. J. Gould and S. Subramani, *Anal. Biochem.* 175:5 (1988; and G. S. A. B. Stewart and P. Williams, *J. Gen. Microbiol.*, 138:1289 (1992).

22. Applicants have generated several other plasmid constructions that may be useful to investigators. These include a pBluescript II KS (+) derivative (TU#65) containing a Kpn I-Eco RI fragment encoding GFP with an Age I site 5' to the translation start and a BAM I site at the termination codon. Also available are gfp versions (TU#60–TU#63) of the four *C. elegans* lacZ expression vectors (pPD16.43, pPD21.28, pPD22.04, and pPD22.11, respectively) described by Fire, et al., 1990 (op. cit.).

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i i i) NUMBER OF SEQUENCES: 1

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

-continued

(i i) MOLECULE TYPE: cDNA

(i i i) HYPOTHETICAL: NO

(v i) ORIGINAL SOURCE:
(A) ORGANISM: Escherichia coli

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ACAAAGGCTA GCAAAGGAGA AGAAC 25

What is claimed is:

1. A host cell comprising a DNA molecule having a regulatory element from a gene, other than a gene encoding an *Aequorea victoria* green-fluorescent protein operatively linked to a DNA sequence encoding the fluorescent *Aequorea victoria* green-fluorescent protein.

2. A cell of claim 1, wherein the cell is selected from a group consisting of bacterial cell, yeast cell, fungal cell, plant cell or animal cell.

3. A cell of claim 1, wherein the regulatory element is a promoter.

4. A cell of claim 3, wherein the promoter is activated by a heavy metal.

5. A cell of claim 3, wherein the promoter is a P450 promoter.

6. A cell of claim 3, wherein the promoter is from a gene encoding a stress protein.

7. A cell of claim 6, wherein the stress protein is a heat-shock protein.

8. A cell of claim 1, wherein the regulatory element is an enhancer.

9. A method for selecting cells expressing a protein of interest which comprises:

(a) introducing into the cells a DNAI molecule having DNA sequence encoding the protein of interest and DNAII molecule having DNA sequence encoding an *Aequorea victoria* green-fluorescent protein;

(b) culturing the introduced cells in conditions permitting expression of the *Aequorea victoria* green-fluorescent protein and the protein of interest; and

(c) selecting the cultured cells which express *Aequorea victoria* green-fluorescent protein, thereby selecting cells expressing the protein of interest,

wherein DNAI and DNAII are linked.

10. A method for selecting cells expressing a protein of interest which comprises:

(a) introducing into the cells a DNAI molecule having DNA sequence encoding the protein of interest and DNAII molecule having DNA sequence encoding an *Aequorea victoria* green-fluorescent protein;

(b) culturing the introduced cells in conditions permitting expression of the *Aequorea victoria* green-fluorescent protein and the protein of interest; and

(c) selecting the cultured cells which express *Aequorea victoria* green-fluorescent protein, thereby selecting cells expressing the protein of interest,

wherein the cells are selected from a group consisting of yeast cells, fungal cells, insect cells, nematode cells, plant or animal cells.

11. A method for localizing a protein of interest in a cell which comprises:

(a) introducing into a cell a DNA molecule having DNA sequence encoding the protein of interest linked to DNA sequence encoding an *Aequorea victoria* green-fluorescent protein such that the protein produced by the DNA molecule will have the protein of interest fused to the *Aequorea victoria* green-fluorescent protein;

(b) culturing the cell in condition permitting expression of the fused protein; and

(c) detecting the location of the fluorescence of the fused protein in the cell, thereby localizing a protein of interest in a cell.

12. A method of claim 11, wherein the cell normally expresses the protein of interest.

* * * * *

09/619,364 #20
Exhibit A

US005777079A

United States Patent [19]

Tsien et al.

[11] Patent Number: 5,777,079

[45] Date of Patent: Jul. 7, 1998

[54] **MODIFIED GREEN FLUORESCENT PROTEINS**

[75] Inventors: **Roger Y. Tsien**, La Jolla; **Roger Heim**, Del Mar, both of Calif.

[73] Assignee: **The Regents of the University of California**, Oakland, Calif.

[21] Appl. No.: **753,143**

[22] Filed: **Nov. 20, 1996**

Related U.S. Application Data

[60] Division of Ser. No. 727,452, filed as PCT/US95/14692, Nov. 13, 1995, which is a continuation-in-part of Ser. No. 337,915, Nov. 10, 1994, Pat. No. 5,625,048.

[51] **Int. Cl.**[6] **C07K 1/00**; C12P 21/04; C12N 15/00; C12N 9/02

[52] **U.S. Cl.** **530/350**; 530/855; 435/69.1; 435/69.7; 435/172.1; 435/189

[58] **Field of Search** 435/91.1, 91.2, 435/172.1, 69.1, 69.7, 189; 530/350, 855

[56] **References Cited**

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5,491,084	2/1996	Chalfie	435/189
5,625,048	4/1997	Tsien et al.	536/23.4

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WO 91/01305	2/1991	WIPO .
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WO 95/07463	3/1995	WIPO .
WO 95/21191	8/1995	WIPO .
WO 96/23810	8/1996	WIPO .
WO 96/23898	8/1996	WIPO .
WO 96/27027	9/1996	WIPO .
WO 96/27675	9/1996	WIPO .
WO 97/11094	3/1997	WIPO .
Wo 97/28261	8/1997	WIPO .

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Heim et al., "Engineering green fluorescent protein for improved brightness, longer wavelengths and fluorescence resonance energy tranfer." *Current Biology* 6(2): 178–182 (1996).

Inouye and Tsuji, "Expression of the gene and fluorescence characteristics of the recombinant protein." *FEBS Letters 341:* 277–280 (1994).

Mitra et al., "Fluorescence resonance energy transfer between blue–emitting and red–shifted excitation derivatives of the green fluorescent protein." *Gene 173(1):* 13–17 (Jul 1, 1996).

Roth, Thesis from the Graduate Program in Biochemistry from Rutgers, The State University of New Jersey (Oct. 1985).

Primary Examiner—Robert A.. Wax
Assistant Examiner—Elizabeth Slobodyansky
Attorney, Agent, or Firm—Fish & Richardson, P.C.

[57] **ABSTRACT**

Modifications in the sequence of Aequorea wild-type GFP provide products having markedly different excitation and emission spectra from corresponding products from wild-type GFP. In one class of modifications, the product derived from the modified GFP exhibits an alteration in the ratio of two main excitation peaks observed with the product derived from wild-type GFP. In another class, the product derived from the modified GFP fluoresces at a shorter wavelength than the corresponding product from wild-type GFP. In yet another class of modifications, the product derived from the modified GFP exhibits only a single excitation peak and enhanced emission relative to the product derived from wild-type GFP.

64 Claims, 7 Drawing Sheets

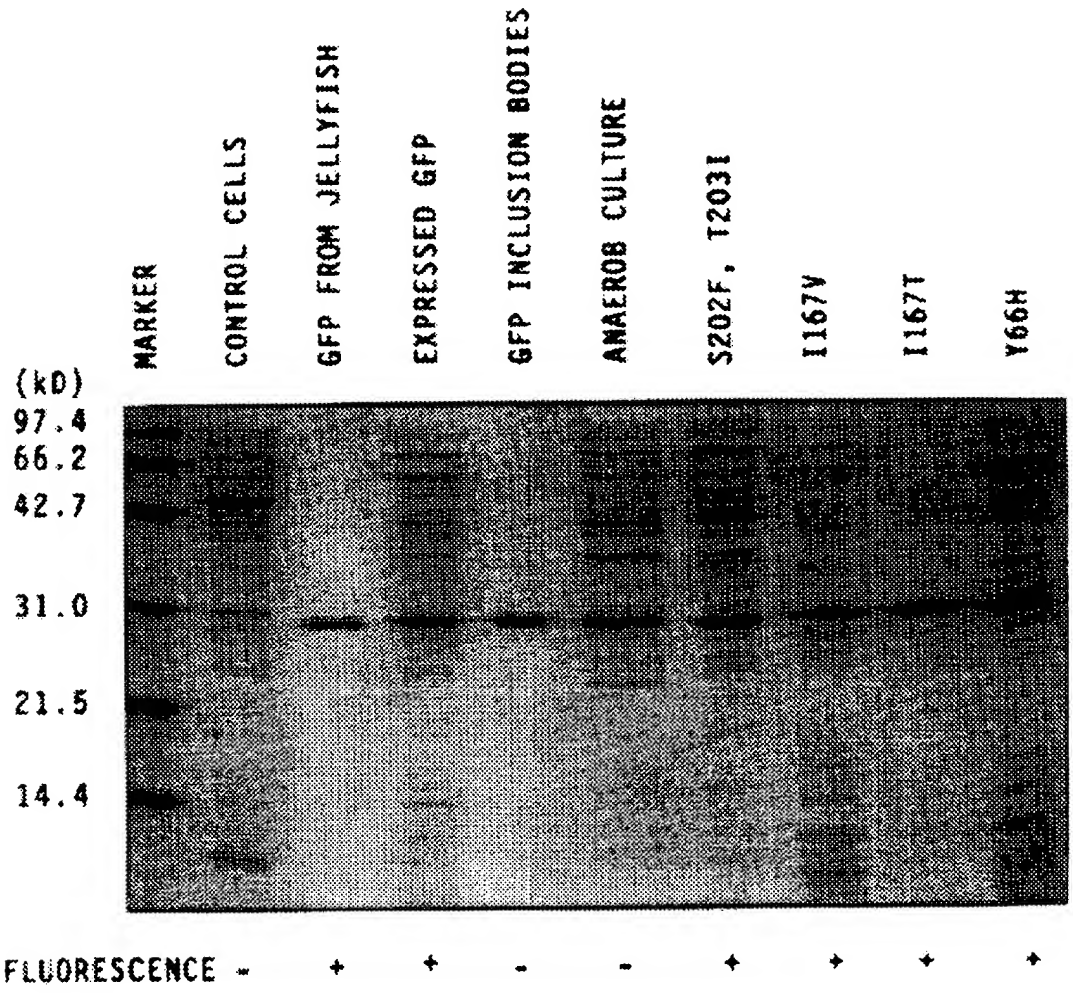

MARKER
CONTROL CELLS
GFP FROM JELLYFISH
EXPRESSED GFP
GFP INCLUSION BODIES
ANAEROB CULTURE
S202F, T203I
I167V
I167T
Y66H
(kD)
97.4
66.2
42.7
31.0
21.5
14.4
FLUORESCENCE - + + - - + + + +

FIG. 1

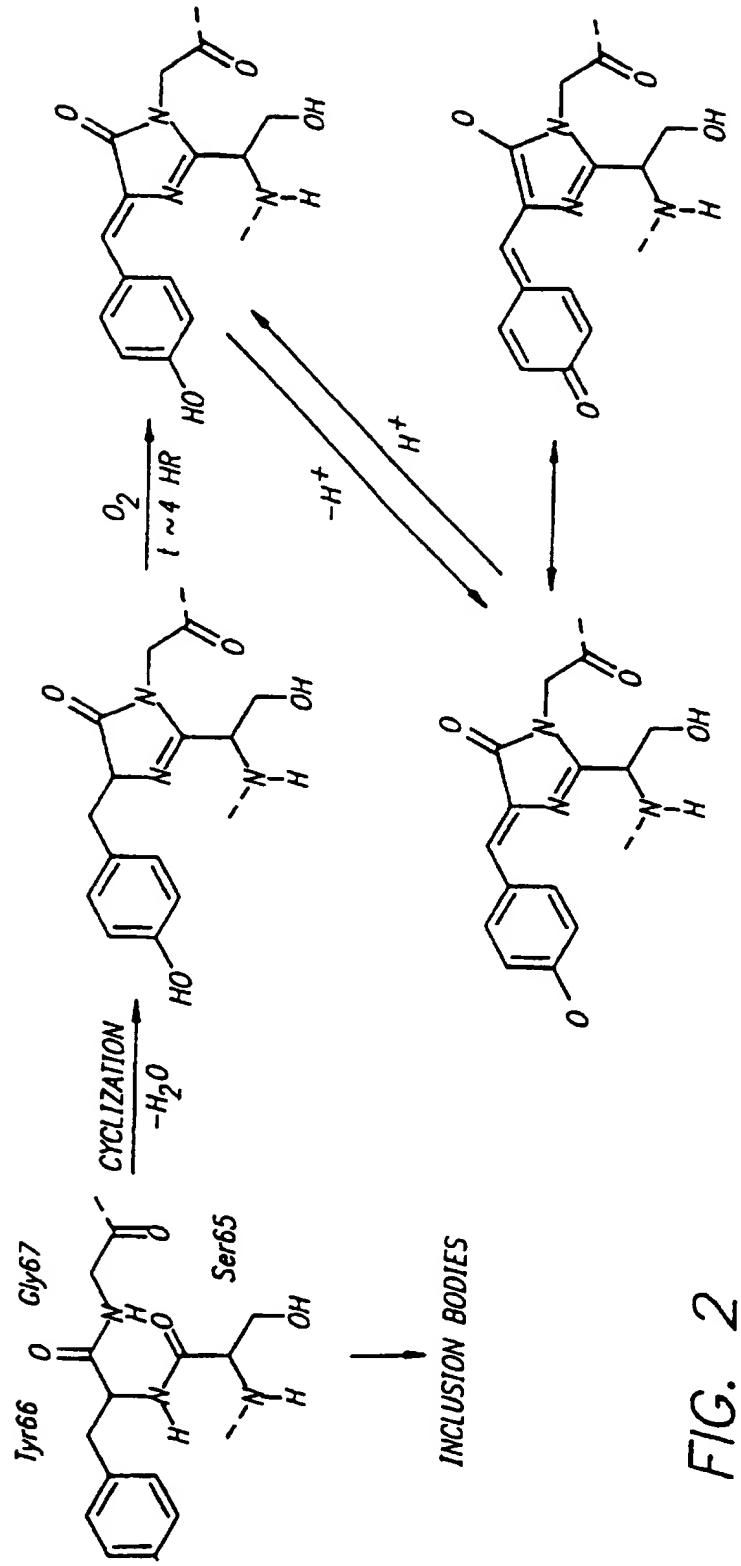

Tyr66
Gly67
Ser65
CYCLIZATION
$-H_2O$
O_2
1~4 HR
$-H^+$
H^+
INCLUSION BODIES

FIG. 2

FIG. 3a

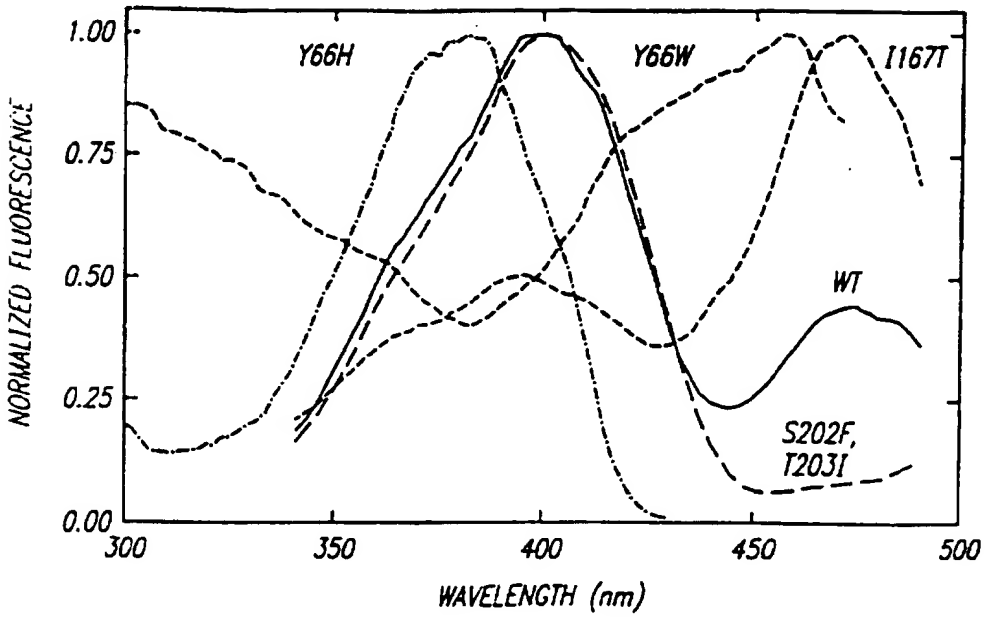

1.00
0.75
0.50
0.25
0.00
NORMALIZED FLUORESCENCE
Y66H
Y66W
I167T
WT
S202F,
T203I
300
350
400
450
500
WAVELENGTH (nm)

FIG. 3b

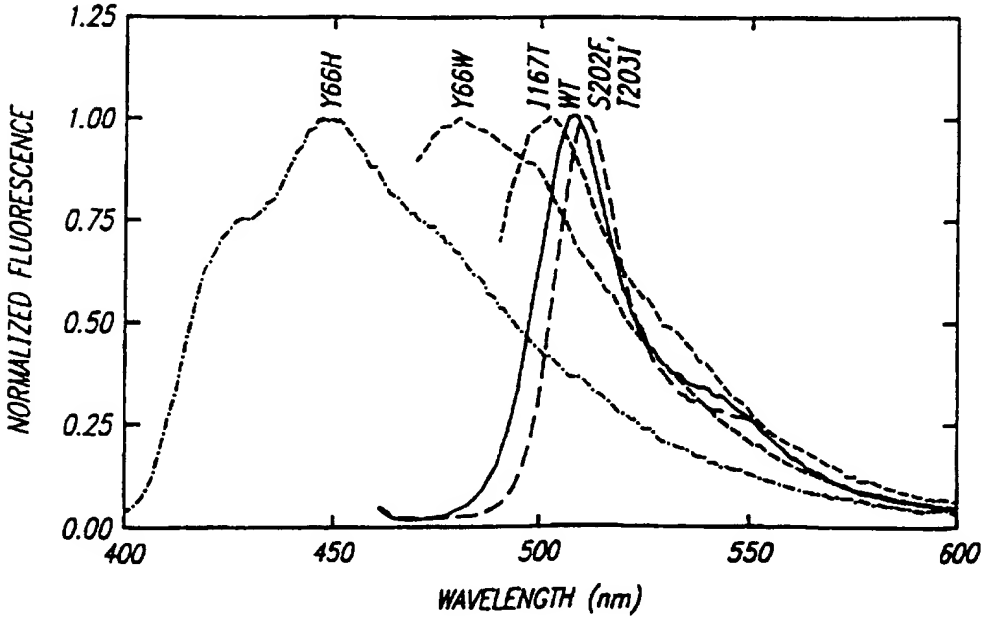

1.25
1.00
0.75
0.50
0.25
0.00
NORMALIZED FLUORESCENCE
Y66H
Y66W
I167T
WT
S202F,
T203I
400
450
500
550
600
WAVELENGTH (nm)

FIG. 4a

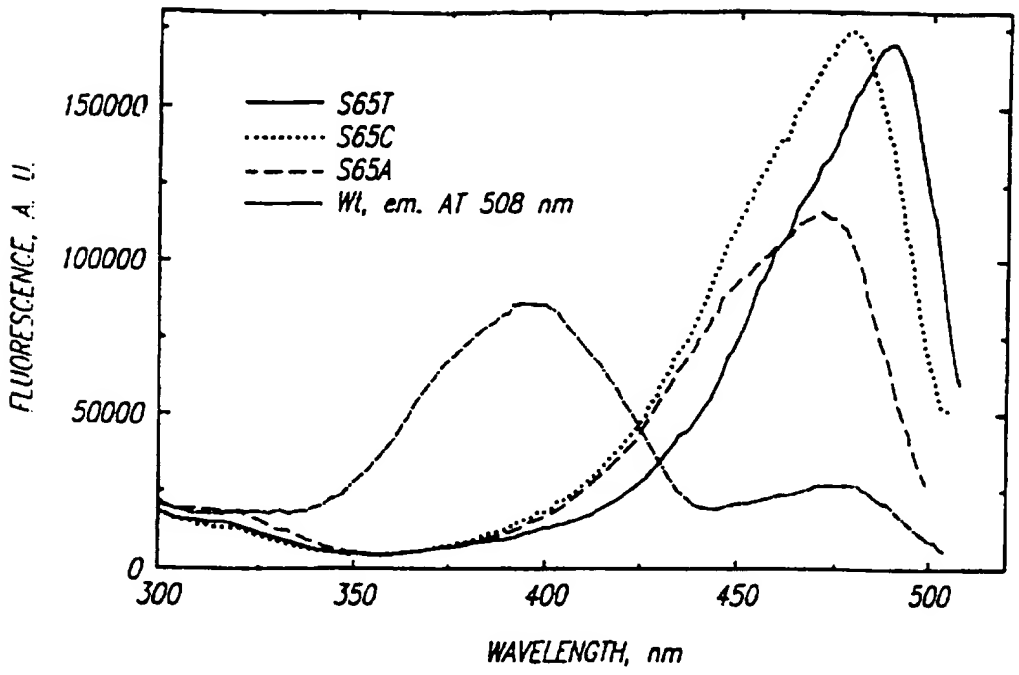

S65T
S65C
S65A
Wt, em. AT 508 nm
FLUORESCENCE, A. U.
150000
100000
50000
0
300
350
400
450
500
WAVELENGTH, nm

FIG. 4b

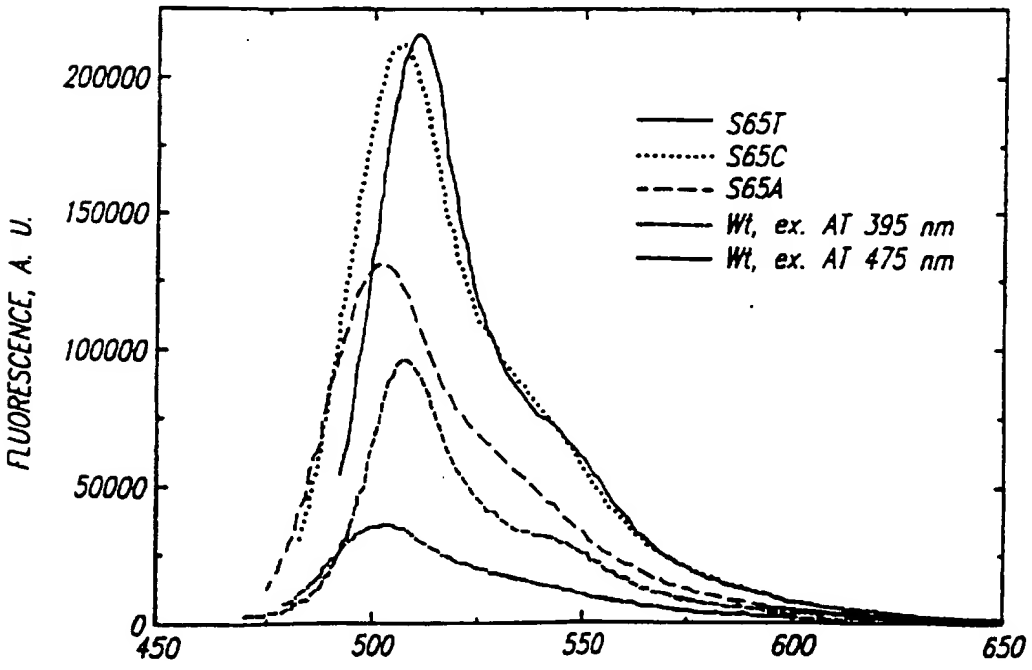

S65T
S65C
S65A
Wt, ex. AT 395 nm
Wt, ex. AT 475 nm
FLUORESCENCE, A. U.
200000
150000
100000
50000
0
450
500
550
600
650

FIG. 5

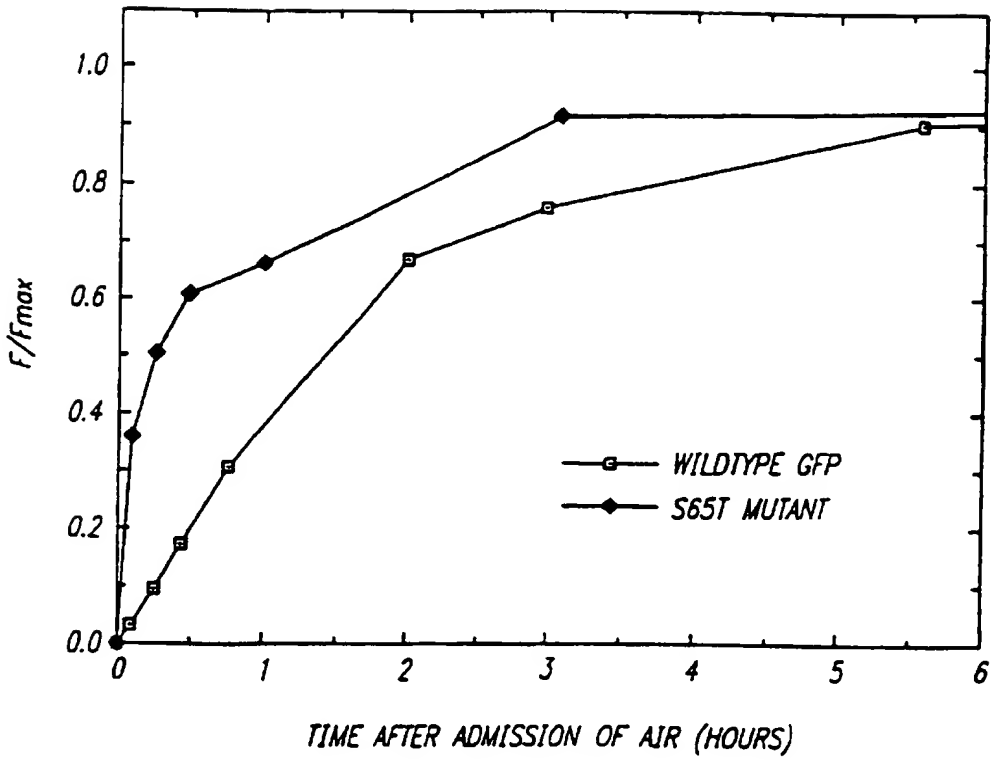

1.0
0.8
0.6
0.4
0.2
0.0
F/Fmax
0
1
2
3
4
5
6
WILDTYPE GFP
S65T MUTANT
TIME AFTER ADMISSION OF AIR (HOURS)

FIG. 6a

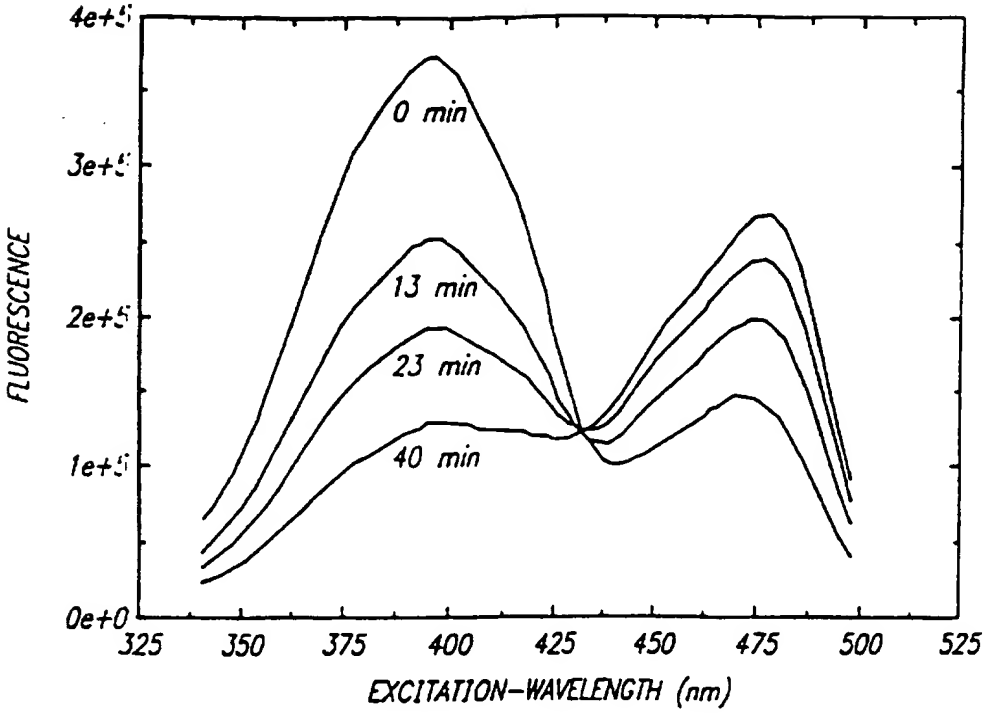
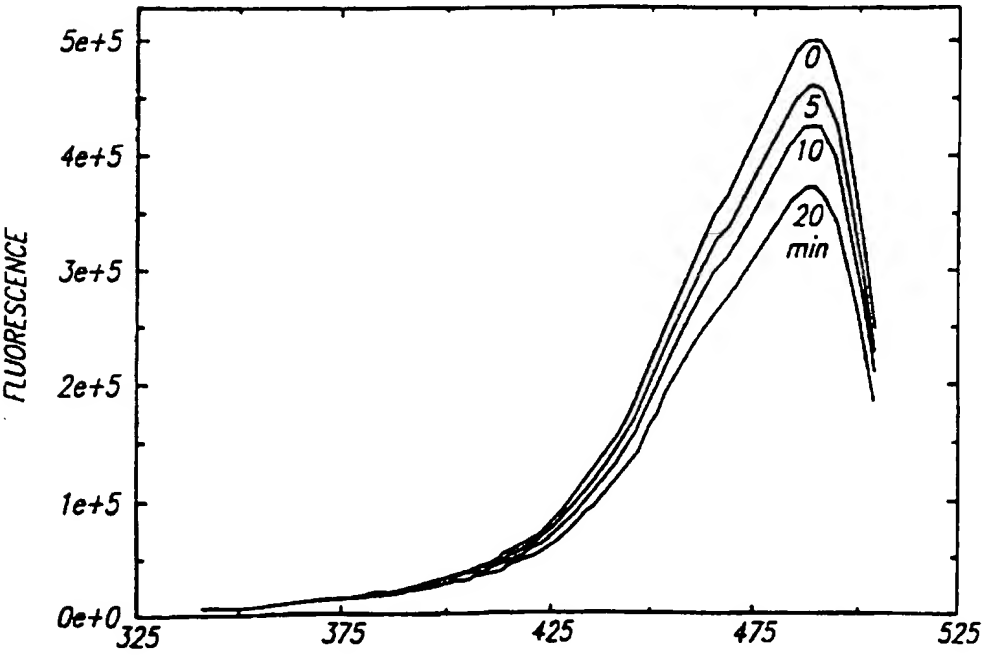

4e+5
3e+5
2e+5
1e+5
0e+0
FLUORESCENCE
0 min
13 min
23 min
40 min
325
350
375
400
425
450
475
500
525
EXCITATION-WAVELENGTH (nm)

FIG. 6b


5e+5
4e+5
3e+5
2e+5
1e+5
0e+0
FLUORESCENCE
0
5
10
20
min
325
375
425
475
525

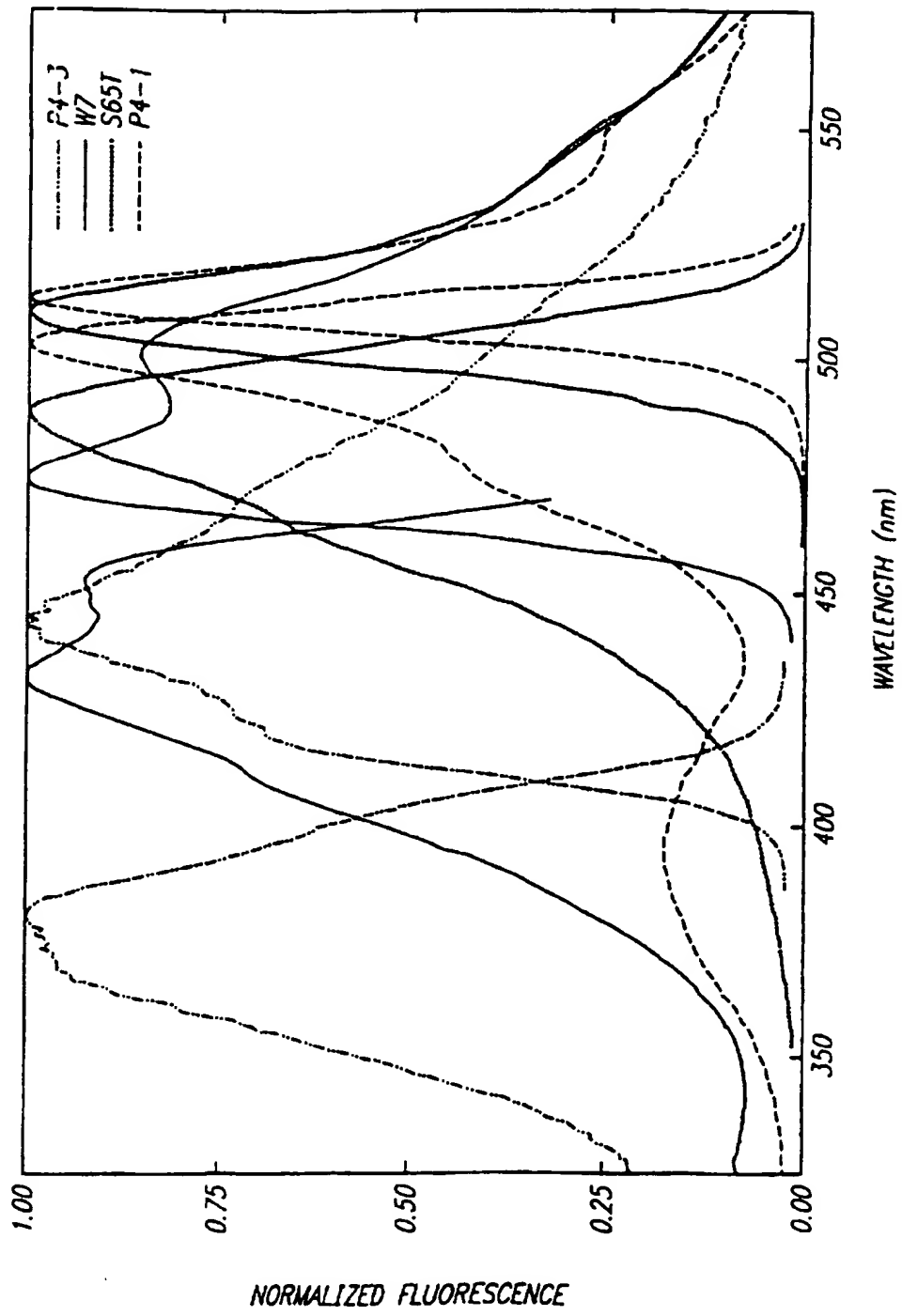

P4-3
W7
S65T
P4-1
1.00
0.75
0.50
0.25
0.00
NORMALIZED FLUORESCENCE
350
400
450
500
550
WAVELENGTH (nm)

FIG. 7

MODIFIED GREEN FLUORESCENT PROTEINS

This is a divisional application of U.S. application Ser. No. 08/727,452, filed Oct. 18, 1996, which is a continuation of PCT/US95/14692, filed Oct. 10, 1995, which is a continuation-in-part of U.S. application Ser. No. 08/337,915, filed Nov. 10,1994, which has issued as U.S. Pat. No. 5,625,048.

The invention was made with Government support under Grant No. NS27177, awarded by the National Institute of Health. The Government has certain rights in this invention.

BACKGROUND OF THE INVENTION

This invention relates generally to the fields of biology and chemistry. More particularly, the invention is directed to modified fluorescent proteins and to methods for the preparation and use thereof.

In biochemistry, molecular biology and medical diagnostics, it is often desirable to add a fluorescent label to a protein so that the protein can be easily tracked and quantified. The normal procedures for labeling requires that the protein be covalently reacted in vitro with fluorescent dyes, then repurified to remove excess dye and any damaged protein. If the labeled protein is to be used inside cells, it usually has to be microinjected; this is a difficult and time-consuming operation that cannot be performed on large numbers of cells. These problems may, however, be eliminated by joining a nucleotide sequence coding for the protein of interest with the sequence for a naturally fluorescent protein, then expressing the fusion protein.

The green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* is a remarkable protein with strong visible absorbance and fluorescence from a p-hdroxybenzylideneimidazolone chromophore, which is generated by cyclization and oxidation of the protein's own Ser-Tyr-Gly sequence at positions 65 to 67. A cDNA sequence [SEQ ID NO:1] for one isotype of GFP has been reported [Prasher, D. C. et al., *Gene* 111, 229–233 (1992)]; cloning of this cDNA has enabled GFP expression in different organisms. The finding that the expressed protein becomes fluorescent in cells from a wide variety of organisms [Chalfie, M. et al., *Science* 263, 802–805 (1994)] makes GFP a powerful new tool in molecular and cell biology and indicates that the oxidative cyclization must be either spontaneous or dependent only on ubiquitous enzymes and reactants.

A major question in protein photophysics is how a single chromophore can give widely different spectra depending on its local protein environment. This question has received the most attention with respect to the multiple colors of visual pigments based on retinal [Merbs, S. L. & Nathans, J. *Science* 258, 46–466 (1992)], but is also important in GFP. The GFP from Aequorea and that of the sea pansy *Renilla reniformis* share the same chromophore, yet Aequorea GFP has two absorbance peaks at 395 and 475 nm, whereas Renilla GFP has only a single absorbance peak at 498 nm, with about 5.5 fold greater monomer extinction coefficient than the major 395 nm peak of the Aequorea protein [Ward, W. W. in *Bioluminescence and Chemiluminescence* (eds. DeLuca, M.A. & McElroy, W. D.) 235–242 (Academic Press, New York, 1981)]. The spectra of the isolated chromophore and denatured protein at neutral pH do not match the spectra of either native protein [Cody, C. W. et al., *Biochemistry* 32, 1212–1218 (1993)].

For many practical applications, the spectrum of Renilla GFP would be preferable to that of Aequorea, because

wavelength discrimination between different fluorophores and detection of resonance energy transfer are easier if the component spectra are tall and narrow rather than low and broad. Furthermore, the longer wavelength excitation peak (475 nm) of Aequorea GFP is almost ideal for fluorescein filter sets and is resistant to photobleaching, but has lower amplitude than the shorter wavelength peak at 395 nm, which is more susceptible to photobleaching [Chalfie et al. (1994), supra]. For all these reasons, it would clearly be advantageous to convert the Aequorea GFP excitation spectrum to a single peak, and preferably at longer wavelengths.

There is also a need in the art for proteins which fluoresce at different wavelengths. Variants of fluorescent proteins with different colors would also be very useful for simultaneous comparisons of multiple protein fates, developmental lineages, and gene expression levels.

Accordingly, it is an object of the present invention to provide improved fluorescent proteins which do not suffer from the drawbacks of native Aequorea GFP.

SUMMARY OF THE INVENTION

In accordance with the present invention, it has been determined that particular modifications in the polypeptide sequence of an Aequorea wild-type GFP [SEQ ID NO:2] lead to formation of products having markedly different excitation and emission spectra from corresponding products derived from wild-type GFP. Visibly distinct colors and/or increased intensities of emission make these products useful in a wide variety of contexts, such as tracking of differential gene expression and protein localization.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention may be better understood with reference to the accompanying drawings, in which:

FIG. 1 compares different versions of GFP by gel electrophoresis and Coomassie blue staining;

FIG. 2 illustrates a proposed biosynthetic scheme for GFP;

FIGS. 3*a* and 3*b* illustrate the excitation and emission spectra of wild-type and a first group of mutant GFPs;

FIGS. 4*a* and 4*b* illustrate the excitation and emission spectra of wild-type and a second group of mutant GFPs;

FIG. 5 illustrates the rate of fluorophore formation in the wild-type GFP and the Ser 65→Thr mutant;

FIGS. 6*a* and 6*b* illustrate the behavior of wild-type GFP and the Ser 65→Thr mutant, respectively, upon progressive irradiation with ultraviolet light; and

FIG. 7 illustrates fluorescence excitation and emission spectra of a third group of GFP mutants.

DETAILED DESCRIPTION OF THE INVENTION

GFP was expressed in *E. coli* under the control of a T7 promoter for quantitative analysis of the properties of the recombinant protein. Gel electrophoresis under denaturing conditions showed protein of the expected molecular weight (27 kDa) as a dominant band (FIG. 1), which could be quantified simply by densitometry of staining with Coomassie blue. Soluble recombinant GFP proved to have identical spectra and the same or even slightly more fluorescence per mole of protein as GFP purified from *Aequorea victoria*, showing that the soluble protein in *E. coli* undergoes correct folding and oxidative cyclization with as high an efficiency as in the jellyfish.

The bacteria also contained inclusion bodies consisting of protein indistinguishable from jellyfish or soluble recombinant protein on denaturing gels (FIG. 1). However, this material was completely non-fluorescent, lacked the visible absorbance bands of the chromophore, and could not be made fluorescent even when solubilized and subjected to protocols that renature GFP [Ward, W. W. & Bokman, S. H., *Biochemistry* 21, 4535–4540 (1982); Surpin, M. A. & Ward, W. W., *Photochem. Photobiol.* 49, Abstract, 25S (1989)]. Therefore, protein from inclusion bodies seemed permanently unable to generate the internal chromophore. An interesting intermediate stage in protein maturation could be generated by growing the bacteria anaerobically. The soluble protein again looked the same as GFP on denaturing gels (FIG. 1) but was non-fluorescent. In this case, fluorescence gradually developed after admission of air, even when fresh protein synthesis was blocked using puromycin and tetracycline. Evidently, the soluble non-fluorescent protein synthesized under anaerobic conditions was ready to become fluorescent once atmospheric oxygen was readmitted. The fluorescence per protein molecule approached its final asymptotic value with a single-exponential time course and a rate constant of $0.24\pm0.06\ hr^{-1}$ (at 22° C.) measured either in intact cells with protein-synthesis inhibitors or in a lysate in which the soluble proteins and cofactors were a thousand fold more dilute. Such pseudo-first order kinetics strongly suggest that no enzymes or cofactors are necessary for the final step of fluorophore formation in GFP.

It has thus been determined that formation of the final fluorophore requires molecular oxygen and proceeds in wild-type protein with a time constant of ~4 h at 22° C. and atmospheric pO_2. This was independent of dilution, implying that the oxidation does not require enzymes or cofactors.

A molecular interpretation is presented in FIG. 2. If the newly translated apoprotein (top left) evades precipitation into inclusion bodies, the amino group of Gly 67 might cyclize onto the carbonyl group of Ser 65 to form an imidazolidin-5-one, where the process would stop (top center) if O_2 is absent. The new N=C double bond would be expected to promote dehydrogenation to form a conjugated chromophore; imidazolidin-5-ones are indeed known to undergo autoxidative formation of double bonds at the 4-position [Kjaer, A. *Acta Chem. Scand.* 7, 1030–1035 (1953); Kidwai, A. R. & Devasia, G. M. *J. Org. Chem.* 27, 4527–4531 (1962)], which is exactly what is necessary to complete the fluorophore (upper right). The protonated and deprotonated species (upper and lower right) may be responsible for the 395 and 470–475 nm excitation peaks, respectively. The excited states of phenols are much more acidic than their ground states, so that emission would come only from a deprotonated species.

The Aequorea GFP cDNA was subjected to random mutagenesis by hydroxylamine treatment or polymerase chain reaction. Approximately six thousand bacterial colonies on agar plates were illuminated with alternating 395 and 475 nm excitation and visually screened for altered excitation properties or emission colors.

According to a first aspect of the present invention, modifications are provided which result in a shift in the ratio of the two excitations peaks of the product after oxidation and cyclization relative to the wild type. Three mutants were found with significant alterations in the ratio of the two main excitation peaks (Table I). The mutations were sequenced and recombined with the wild-type gene in different ways to eliminate neutral mutations and assign the fluorescence effects to single amino acid substitutions, except for H9 where two neighboring mutations have not yet been separated. They all lay in the C terminal part of the protein (Table I), remote in primary sequence from the chromophore formed from residues 65–67.

These and other modifications are defined herein with reference to the amino acid sequence [SEQ ID NO:2] encoded by the reported cDNA [SEQ ID NO:1]; the first amino acid identified is the one found at the indicated location in the reported sequence, while the second indicates the substitution found in the modified form. The fluorescent product derived from a wild-type or modified GFP polypeptide sequence is no longer strictly speaking a simple polypeptide after oxidation and cyclization; however, reference is sometimes made for sake of simplicity herein to the polypeptide (e.g., "wild-type GFP" or "modified GFP") where what is intended would be obvious from the context. Compared with wild-type GFP, H9 (Ser 202→Phe, Thr 203→Ile) had increased fluorescence at 395 nm excitation; P9 (Ile 167→Val) and P11 (Ile 167→Thr) were more fluorescent at 475 nm excitation.

One possibility for these spectral perturbations in P9 and P11 is that the mutations at Ile 167 shift a positive charge slightly closer to the phenolic group of the fluorophore; this should both increase the percentage of phenolic anion, which is probably the species responsible for the 470–475 nm excitation peak, and shift the emission peak hypsochromically. However, the hypothesized ionizable phenolic group would have to be buried inside the protein at normal pH, because the ratio of 471 to 396 nm peaks in the mutants could not be further affected by external pH until it was raised to 10, just below the threshold for denaturation. The pH-sensitivity of wild-type GFP is similar [Ward, W. W. et al., *Photochem. Photobiol.* 35, 803–808 (1982)].

According to another aspect of the invention, a mutant P4 (Tyr 66→His) was identified which was excitable by ultraviolet and fluoresced bright blue in contrast to the green of wild type protein. The excitation and emission maxima were hypsochromically shifted by 14 and 60 nm respectively from those of wild-type GFP. The mutated DNA was sequenced and found to contain five amino acid substitutions, only one of which proved to be critical: replacement of Tyr 66 in the center of the chromophore by His (corresponding to a change in the GFP cDNA sequence [SEQ ID NO:1] at 196–198 from TAT to CAT).

The surprising tolerance for substitution at this key residue prompted further site-directed mutagenesis to Trp and Phe at this position. Trp gave excitation and emission wavelengths intermediate between Tyr and His (Table I) but was only weakly fluorescent, perhaps due to inefficiency of folding or chromophore formation due to steric considerations. Phe gave weak fluorescence with an excitation maximum at 358 nm and an emission maximum at 442 nm. Accordingly, pursuant to this aspect of the invention modified GFP proteins which fluoresce at different wavelengths (preferably, different by at least 10 nm and more preferably by at least 50 nm) relative to the native protein are provided for example, those wherein Tyr 66 is replaced by Phe, His or Trp.

In a further embodiment of this aspect of the invention, a double mutant Y66H, Y145F was identified which had almost the same wavelengths as the single mutant Y66H but almost twice the brightness, due mainly to a higher quantum efficiency of fluorescence. The double mutant also developed its fluorescence during overnight growth, whereas the single mutant required several days.

In accordance with further embodiments of this aspect of the invention, a first round of mutagenesis to increase the

brightness of Y66W yielded M153T/V163A/N212K as additional substitutions. This mutant was subjected to another round of mutagenesis, resulting in two further sets, N146I and I123V/Y145H/H148R (Table II). The quantum efficiency of these mutants is now comparable to wild-type GFP. The clustering of the substitutions in residues 145 to 163 suggest that those residues lie relatively close to the chromophore and that reductions in the size of their side chains might be compensating for the larger size of tryptophan compared to tyrosine.

Pursuant to yet another aspect of the present invention, modified GFP proteins are provided which provide substantially more intense fluorescence per molecule than the wild type protein. Modifications at Ser 65 to Ala, Ieu, Cys, Val, Ile or Thr provide proteins with red-shifted and brighter spectra relative to the native protein. In particular, the Thr mutant (corresponding to a change in the GFP cDNA sequence |SEQ ID NO:1| at 193–195 from TCT to ACT) and Cys mutant (corresponding to a change in the GFP cDNA sequence |SEQ ID NO:1| at 193–195 from TCT to TGT) are about six times brighter than wild type when excited at the preferred long-wavelength band above 450 nm. As a consequence, these modified proteins are superior to wild type proteins for practically all applications. Further, the brightness of these modified proteins matches the brightness reported in the literature for Renilla GFP; thus, these proteins clearly obviate the objections to the dimness of Aequorea GFP. In fact, it is speculated that the chromophores in these modified proteins may exhibit the optimum brightness which could be achieved with a general structure derived from the Aequorea GFP chromophore. In particular, these mutations provide products exhibiting one or more of the following salient characteristics which distinguish them clearly over the corresponding product from a wild-type GFP: reduced efficiency of excitation by wavelengths between about 350 and 420 nm; enhanced excitation and emission efficiency when excited with wavelengths longer than about 450 nm; increased resistance to light-induced shifts in the excitation spectrum; and faster kinetics of fluorophore generation. In contrast, mutations to Trp, Arg, Asn, Phe and Asp did not provide improved brightness.

Mutagenesis of S65T to shift its wavelengths further to the red yielded M153A/K238E (Table II) as the GFP variant with the longest-wavelength excitation maximum yet described, 504 nm vs. 490 nm for S65T. Surprisingly, the emission peak hardly changed (514 nm vs. 511 nm), so that the separation between the excitation and emission peaks (Stokes' shift) is extremely narrow, only 10 nm. This is one of the smallest values reported for any fluorophore in aqueous solution at room temperature. As in the Y66W series, M153 seems to be influential. It is doubtful that K238E is important, because this substitution has been found to be without effect in other mutants.

As would be readily apparent to those working in the field, to provide the desired fluorescent protein it would not be necessary to include the entire sequence of GFP. In particular, minor deletions at either end of the protein sequence are expected to have little or no impact on the fluorescence spectrum of the protein. Therefore, by a mutant or wild-type GFP sequence for purposes of the present invention are contemplated not only the complete polypeptide and oligonucleotide sequences discussed herein, but also functionally-equivalent portions thereof (i.e., portions of the polypeptide sequences which exhibit the desired fluorescence properties and oligonucleotide sequences encoding these polypeptide sequences). For example, whereas the chromophore itself (position 65–67) is obviously crucial, the locations of known neutral mutations suggest that amino acids 76–115 are less critical to the spectroscopic properties of the product. In addition, as would be immediately apparent to those working in the field, the use of various types of fusion sequences which lengthen the resultant protein and serve some functional purpose in the preparation or purification of the protein would also be routine and are contemplated as within the scope of the present invention. For example, it is common practice to add amino acid sequences including a polyhistidine tag to facilitate purification of the product proteins. As such fusions do not significantly alter the salient properties of the molecules comprising same, modified GFPs as described herein including such fusion sequences at either end thereof are also clearly contemplated as within the scope of the present invention.

Similarly, in addition to the specific mutations disclosed herein, it is well understood by those working in the field that in many instances modifications in particular locations in the polypeptide sequence may have no effect upon the properties of the resultant polypeptide. Unlike the specific mutations described in detail herein, other mutations provide polypeptides which have properties essentially or substantially indistinguishable from those of the specific polypeptides disclosed herein. For example, the following substitutions have been found to be neutral (i.e., have no significant impact on the properties of the product): Lys 3→Arg; Asp 76→Gly; Phe 99→Ile; Asn 105→Ser; Glu 115→Val; Thr 225→Ser; and Lys 238→Glu. These equivalent polypeptides (and oligonucleotide sequences encoding these polypeptides) are also regarded as within the scope of the present invention. In general, the polypeptides and oligonucleotide sequences of the present invention (in addition to containing at least one of the specific mutations identified herein) will be at least about 85 % homologous, more preferably at least about 90% homologous, and most preferably at least about 95% homologous, to the wild-type GFP described herein. Because of the significant difference in properties observed upon introduction of the specified modifications into a GFP sequence, the presence of the specified modifications relative to the corresponding reported sequence for wild-type GFP |SEQ ID NO:2| are regarded as central to the invention.

The oligonucleotide sequences of the present invention are particularly useful in processes for labelling polypeptides of interest, e.g., by the construction of genes encoding fluorescent fusion proteins. Fluorescence labeling via gene fusion is site-specific and eliminates the present need to purify and label proteins in vitro and microinject them into cells. Sequences encoding the modified GFPs of the present invention may be used for a wide variety of purposes as are well known to those working in the field. For example, the sequences may be employed as reporter genes for monitoring the expression of the sequence fused thereto; unlike other reporter genes, the sequences require neither substrates nor cell disruption to evaluate whether expression has be achieved. Similarly, the sequences of the present invention may be used as a means to trace lineage of a gene fused thereto during the development of a cell or organism. Further, the sequences of the present invention may be used as a genetic marker; cells or organisms labeled in this manner can be selected by, e.g., fluorescence-activated cell sorting. The sequences of the present invention may also be used as a fluorescent tag to monitor protein expression in vivo, or to encode donors or acceptors for fluorescence resonance energy transfer. Other uses for the sequences of the present invention would be readily apparent to those

working in the field, as would appropriate techniques for fusing a gene of interest to an oligonucleotide sequence of the present invention in the proper reading frame and in a suitable expression vector so as to achieve expression of the combined sequence.

The availability of several forms of GFP with such different spectral properties should facilitate two-color assessment of differential gene expression, developmental fate, or protein trafficking. For example, if one wanted to screen for a drug that is specific to activate expression of gene A but not gene B, one could fuse the cDNA for one color of GFP to the promoter region of gene A and fuse the cDNA for another color to the promoter region of gene B. Both constructs would be transfected into target cells and the candidate drugs could be assayed to determine if they stimulate fluorescence of the desired color, but not fluorescence of the undesired color. Similarly, one could test for the simultaneous expression of both A and B by searching for the presence of both colors simultaneously.

As another example, to examine the precise temporal or spatial relationship between the generation or location of recombinant proteins X and Y within a cell or an organism, one could fuse genes for different colors of GFP to the genes for proteins X and Y, respectively. If desired, DNA sequences encoding flexible oligopeptide spacers could be included to allow the linked domains to function autonomously in a single construct. By examining the appearance of the two distinguishable colors of fluorescence in the very same cells or organisms, one could compare and contrast the generation or location of the proteins X and Y with much greater precision and less biological variability than if one had to compare two separate sets of cells or organisms, each containing just one color of GFP fused to either protein X or Y. Other examples of the usefulness of two colors would be obvious to those skilled in the art.

The further mutations to brighten the Y66H and Y66W variants of GFP enhance the possibility of using two or three colors of fluorescent protein to track differential gene expression, protein localizations or cell fates. For example, mutants P4-3 (Y66H/Y145F), W7 (Y66W/N146I/M153T/V163A/N212K) and S65T can all be distinguished from each other. P4-3 is specifically detected by exciting at 290–370 nm and collecting emission at 420–460 nm. W7 is specifically detected by exciting at 410–457 nm and collecting emission at 465–495 nm. S65T is specifically detected by exciting at 483–493 nm and collecting emission at wavelengths greater than 510 nm. Bacteria carrying these three proteins are readily discriminated under a microscope using the above wavelength bandpass filters.

The chromophore in GFP is well buried inside the rest of the protein, so much of the dimness of the original point mutants was presumably due to steric mismatch between the substituted amino acid and the cavity optimized for tyrosine. The location of the beneficial mutations implies that residues 145–163 are probably close to the chromophore. The M153A/S65T mutant has the longest wavelengths and smallest Stokes' shift of any known fluorescent protein that does not use a cofactor.

The invention may be better understood with reference to the accompanying examples, which are intended for purposes of illustration only and should not be construed as in any sense limiting the scope of the invention as defined by the claims appended hereto.

EXAMPLE 1

The coding region of GFP clone 10.1 [Prasher et al. (1992), supra] was amplified by PCR to create NdeI and BamHI sites at the 5'and 3'ends, respectively, and was cloned behind the T7 promoter of pGEMEX2 (Promega) replacing most of the T7 gene 10. The resulting plasmid was transformed into the strain JM109(DE3) (Promega Corp., Madison, Wis.), and high level expression was achieved by growing the cultures at 24° C. to saturation without induction by IPTG. To prepare soluble extracts, 1.5 ml cell suspension were collected, washed and resuspended in 150 µl 50 mM Tris/HCl, pH 8.0, 2 mM EDTA. Lysozyme and DNAse I were added to 0.2 mg/ml and 20 µg/ml, respectively, and the samples were incubated on ice until lysis occurred (1–2 hours). The lysates were then clarified by centrifuging at 12,000×g for 15 minutes. Inclusion bodies were obtained as described in the literature [Sambrook, J. et al. in *Molecular Cloning: A Laboratory Manual* Vol. 2, 17.37–17.41 (Cold Spring Harbor Press, Cold Spring Harbor, New York, 1989)].

As illustrated in FIG. 1, soluble extracts of *E. coli* expressing GFP show a predominant band which is absent in extracts from control cells and has the same electrophoretic mobility as native GFP isolated from the jellyfish *A. Victoria*. Inclusion bodies of expressing cells consist mainly of non-fluorescent GFP which has the same mobility as soluble GFP. Non-fluorescent soluble GFP of anaerobically grown cultures is also a major band with correct mobility. Soluble extracts of the mutated clones H9, P9, P11 and P4 again contain a dominant protein with essentially the same molecular weight.

Random mutagenesis of the GFP cDNA was done by increasing the error rate of the polymerase chain reaction with 0.1 mM $MnCl_2$, 50 µM dATP and 200 µM of dGTP, dCTP, and dTTP [Muhlrad, D. et al., Yeast 8, 79–82 (1992)]. The product was ligated into pGEMEX2 and subsequently transformed into JM109(DE3). Colonies on agar were visually screened for different emission colors and ratios of brightness when excited at 475 vs. 395 nm.

FIGS. 3*a* and 3*b* illustrate the excitation and emission spectra of wild-type and mutant GFPs. In FIGS. 3*a* and 3*b*, -- wild-type; - - S202F, T203I; - - - I167T; - - - - - Y66W; -●- ●Y66H. Samples were soluble fractions from *E. coli* expressing the proteins at high level, except for Y66W, which was obtained in very low yield and measured on intact cells. Autofluorescence was negligible for all spectra except those of Y66W, whose excitation spectrum below 380 nm may be contaminated by autofluorescence. Excitation and emission spectra were measured with 1.8 nm bandwidths and the non-scanning wavelength set to the appropriate peak. Excitation spectra were corrected with a rhodamine B quantum counter, while emission spectra (except for Y66W) were corrected for monochromator and detector efficiencies using manufacturer-supplied correction spectra. All amplitudes have been arbitrarily normalized to a maximum value of 1.0. A comparison of brightness at equal protein concentrations is provided in Table I.

TABLE I

Characteristics of mutated vs. wild-type GFP

Variant	Mutation	Excitation Maxima (nm)[a]	Emission Maxima (nm)[b]	Relative[c] Fluorescence
Wild type	none	396 (476)	508 (503)	(≡100%)
H9	Ser 202→Phe, Thr 203→Ile	398	511	117%[d]
P9	Ile 167→Val	471 (396)	502 (507)	166%[e]

TABLE I-continued

Characteristics of mutated vs. wild-type GFP

Variant	Mutation	Excitation Maxima (nm)[a]	Emission Maxima (nm)[b]	Relative[c] Fluorescence
P11	Ile 167→Thr	471 (396)	502 (507)	188%[e]
P4	Tyr 66→His	382	448	57%[f]
W	Tyr 66→Trp	458	480	n.d.

[a]Values in parentheses are lower-amplitude peaks.
[b]Primary values were observed when exciting at the main excitation peak; values in parentheses were observed when illuminating at the lower-amplitude excitation peak.
[c]Equal amounts of protein were used based on densitometry of gels stained with Coomassie Blue (Fig. 1).
[d]Emission maxima of spectra recorded at excitation 395 nm were compared.
[e]Emission maxima of spectra recorded at excitation 475 nm were compared.
[f]Emission spectrum of P4 recorded at 378 nm excitation was integrated and compared to the integrated emission spectrum of wild type recorded at 475 nm excitation; both excitation and emission characteristics were corrected.

EXAMPLE 2

Oligonucleotide-directed mutagenesis at the codon for Ser-65 of GFP cDNA was performed by the literature method [Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. USA* 82, 488] using the Muta-Gene Phagemid in Vitro Mutagenesis Kit version 2, commercially available from Bio-Rad, Richmond, Calif. The method employs a bacterial host strain deficient for dUTPase (dut) and uracil-N-glycosylase (ung), which results in an occasional substitution of uracil for thymine in newly-synthesized DNA. When the uracil-containing DNA is used as a wild-type template for oligonucleotide-directed in vitro mutagenesis, the complementary (mutant) strand can be synthesized in the presence of deoxynucleotides, ligase and polymerase using the mutagenic oligonucleotide to prime DNA synthesis; the Version 2 kit utilizes unmodified T7 DNA polymerase to synthesize the complementary strand. When the heteroduplex molecule is transformed into a host with an active uracil-N-glycosylase (which cleaves the bond between the uracil base and the ribose molecule, yielding an apyrimidic site), the uracil-containing wild-type strand is inactivated, resulting in an enrichment of the mutant strand.

The coding region of GFP cDNA was cloned into the BamHI site of the phagemid pRSET$_B$ from Invitrogen (San Diego, Calif.). This construct was introduced into the dut, ung double mutant *E. coli* strain CJ236 provided with the Muta-Gene kit and superinfected with helper phage VCSM13 (Stratagene, La Jolla, Calif.) to produce phagemid particles with single-stranded DNA containing some uracils in place of thymine. The uracil-containing DNA was purified to serve as templates for in vitro synthesis of the second strands using the mutagenic nucleotides as primers. The DNA hybrids were transformed into the strain XL1blue (available from Stratagene), which has a functional uracil-N-glycosylase; this enzyme inactivates the parent wild-type DNA strand and selects for mutant clones. DNA of several colonies were isolated and checked for proper mutation by sequencing.

To express the mutant proteins, the DNA constructs obtained by mutagenesis were transformed into *E. coli* strain BL21(DE3)LysS (Novagen, Madison, Wis.), which has a chromosomal copy of T7 polymerase to drive expression from the strong T7 promotor. At room temperature 3 ml cultures were grown to saturation (typically, overnight) without induction. Cells from 1 ml of culture were collected, washed and finally resuspended in 100 µl of 50 mM Tris pH 8.0, 300 mM NaCl. The cells were then lysed by three cycles of freeze/thawing (liquid nitrogen/30° C. water bath). The soluble fraction was obtained by pelletting cell debris and unbroken cells in a microfuge.

To facilitate purification of the recombinant proteins, the vector used fuses a histidine tag (6 consecutive His) to the N-terminus of the expressed proteins. The strong interaction between histidine hexamers and Ni^{2+}ions permitted purification of the proteins by NI-NTA resin (available commercially from Qiagen, Chatsworth, Calif.). Microcolumns (10 µl bed volume) were loaded with 100 µl soluble extract (in 50 mM Tris pH 8.0, 300 mM NaCl), washed with 10 bed volumes of the same buffer and with 10 volumes of the buffer containing 20 mM imidazole. The recombinant proteins were then eluted with the same buffer containing 100 mM imidazole.

Aliquots of the purified mutant GFP proteins were run along with wild-type GFP on a denaturing polyacrylamide gel. The gel was stained with Coomassie blue and the protein bands were quantified by scanning on a densitometer. Based on these results, equal amounts of each version of protein were used to run fluorescence emission and excitation spectra.

FIGS. 4*a* and 4*b* compare the excitation and emission spectra of wild-type and Ser 65 mutants. In FIG. 4*a*, --S65T; - - S65A; - - - S65C; -●-● wild-type (emission at 508 nm). In FIG. 4B, -- S65T; - - S65A; - - - S65C; ●●● wild-type (excitation at 395 nm); -●-● wild-type (excitation at 475 nm). Excitation and emission spectra were measured with 1.8 nm bandwidths and the non-scanning wavelength set to the appropriate peak. As is apparent from FIG. 4*b*, all three mutants exhibited substantially higher intensity of emission relative to the wild-type protein.

FIG. 5 illustrates the rates of fluorophore formation in wild-type GFP and in the Ser 65→Thr mutant. *E. coli* expressing either wild-type or mutant GFP were grown anaerobically. At time=0, each sample was exposed to air; further growth and protein synthesis were prevented by transferring the cells to nutrient-free medium also containing sodium azide as a metabolic inhibitor. Fluorescence was subsequently monitored as a function of time. For each culture, the fluorescence intensities are expressed as a fraction of the final fluorescence intensity obtained at t=18 to 20 hours, after oxidation had proceeded to completion. From FIG. 5, it is apparent that development of fluorescence proceeds much more quickly in the mutant than in wild-type GFP, even after normalization of the absolute brightnesses (FIGS. 4*a* and 4*b*). Therefore, when the development of GFP fluorescence is used as an assay for promotor activation and gene expression, the mutant clearly gives a more rapid and faithful measure than wild-type protein.

FIGS. 6*a* and 6*b* illustrate the behavior of wild-type GFP and the Ser 65→Thr mutant, respectively, upon progressive irradiation with ultraviolet light. Numbers indicate minutes of exposure to illumination at 280 nm; intensity was the same for both samples. Wild-type GFP (FIG. 6*a*) suffered photoisomerization, as shown by a major change in the shape of the excitation spectrum. Illumination with broad band (240–400 nm) UV caused qualitatively similar behavior but with less increase of amplitude in the 430–500 nm region of the spectrum. The photoisomerization was not reversible upon standing in the dark. This photoisomerization would clearly be undesirable for most uses of wild-type GFP, because the protein rapidly loses brightness when excited at its main peak near 395 nm. The mutant (FIG. 6*b*) showed no such photoisomerization or spectral shift.

EXAMPLE 3

GFP cDNAs encoding for Tyr66→His (Y66H), Tyr66→Trp (Y66W), or Ser65→Thr (S65T) were separately

further mutagenized by the polymerase chain reaction and transformed into *E. coli* for visual screening of colonies with unusual intensities or colors. Isolation, spectral characterization (Table II and FIG. 7), and DNA sequencing yielded several additional useful variants.

Random mutagenesis of the gfp cDNA was done by increasing the error rate of the PCR with 0.1 mM $MnCl_2$ and unbalanced nucleotide concentrations. The GFP mutants S65T, Y66H and Y66W had been cloned into the BamH1 site of the expression vector pRSETB (Invitrogen), which includes a T7 promoter and a polyhistidine tag. The GFP coding region (shown in bold) was flanked by the following 5' and 3' sequences: 5'-G GAT CCC CCC GCT GAA TTC ATG . . . AAA TAA TAA GGA TCC-3'. The 5' primer for the mutagenic PCR was the T7 primer matching the vector sequence; the 3' primer was 5'-GGT AAG CTT TTA TTT GTA TAG TTC ATC CAT GCC-3', specific for the 3' end of GFP, creating a HindIII restriction site next to the stop codon. Amplification was over 25 cycles (1 min at 94° C., 1 min 52° C., 1 min 72° C.) using the AmpliTaq polymerase from Perkin Elmer. Four separate reactions were run in which the concentration of a different nucleotide was lowered from 200 μM to 50 μM. The PCR products were combined, digested with BamHI and HindIII and ligated to the pRSETB cut with BamHI and HindIII. The ligation mixture was dialyzed against water, dried and subsequently transformed into the bacterial strain BL21(DE3) by electroporation (50 μl electrocompetent cells in 0.1 cm cuvettes, 1900 V, 200 ohm, 25 μF). Colonies on agar were visually screened for brightness as previously described herein. The selected clones were sequenced with the Sequenase version 2.0 kit from Unites States Biochemical.

Cultures with freshly transformed cells were grown at 37° C. to an optical density of 0.8 at 600 nm, then induced with 0.4 mM isopropylthiogalactoside overnight at room temperature. Cells were washed in PBS pH 7.4, resuspended in 50 mM Tris pH 8.0, 300 mM NaCl and lysed in a French press. The polyhistidine-tagged GFP proteins were purified from cleared lysates on nickel-chelate columns (Qiagen) using 100 mM imidazole in the above buffer to elute the protein.

Excitation spectra were obtained by collecting emission at the respective peak wavelengths and were corrected by a Rhodamine B quantum counter. Emission spectra were likewise measured at the respective excitation peaks and were corrected using factors from the fluorometer manufacturer (Spex Industries, Edison, N.J.). In cleavage experiments emission spectra were recorded at excitation 368 nm. For measuring molar extinction coefficients, 20 to 30 μg of protein were used in 1 ml of PBS pH 7.4. Quantum yields of wild-type GFP, S65T, and P4-1 mutants were estimated by comparison with fluorescein in 0.1 N NaOH as a standard of quantum yield 0.91 [ed. Miller, J. N., *Standards in Fluorescence Spectrometry* (Chapman and Hall, New York, 1981)]. Mutants P4 and P4-3 were likewise compared to 9-amino-acridine in water (quantum yield 0.98). W2 and W7 were compared to both standards, which fortunately gave concordant results.

FIG. 7 illustrates the fluorescence excitation and emission spectra of different GFP mutants. All spectra were normalized to a maximal value of 1. Each pair of excitation and emission spectrum is depicted by a distinct line style.

The fluorescence properties of the obtained GFP mutants are reported in Table II.

TABLE II

Fluorescence properties of GFP mutants

Clone	Mutations	Excitation max (nm)	Emission max (nm)	Extinct. Coeff. ($M^{-1}cm^{-1}$)	Quantum yield
P4-3	Y66H Y145F	381	445	14,000	0.38
W7	Y66W N146I M153T V163A N212K	433 (453)	475 (501)	18,000 (17,100)	0.67
W2	Y66W I123V Y145H H148R M153T V163A N212K	432 (453)	480	10,000 (9,600)	0.72
P4-1	S65T M153A K238E	504 (396)	514	14,500 (8,600)	0.54

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i i i) NUMBER OF SEQUENCES: 2

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 716 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(i x) FEATURE:
(A) NAME/KEY: CDS

-continued

(B) LOCATION: 1..716

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
ATG AGT AAA GGA GAA GAA CTT TTC ACT GGA GTT GTC CCA ATT CTT GTT          48
Met Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu Val
  1               5                  10                  15

GAA TTA GAT GGT GAT GTT AAT GGG CAC AAA TTT TCT GTC AGT GGA GAG          96
Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly Glu
            20                  25                  30

GGT GAA GGT GAT GCA ACA TAC GGA AAA CTT ACC CTT AAA TTT ATT TGC         144
Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile Cys
        35                  40                  45

ACT ACT GGA AAA CTA CCT GTT CCA TGG CCA ACA CTT GTC ACT ACT TTC         192
Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr Phe
    50                  55                  60

TCT TAT GGT GTT CAA TGC TTT TCA AGA TAC CCA GAT CAT ATG AAA CAG         240
Ser Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys Gln
65                  70                  75                  80

CAT GAC TTT TTC AAG AGT GCC ATG CCC GAA GGT TAT GTA CAG GAA AGA         288
His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu Arg
                85                  90                  95

ACT ATA TTT TTC AAA GAT GAC GGG AAC TAC AAG ACA CGT GCT GAA GTC         336
Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu Val
            100                 105                 110

AAG TTT GAA GGT GAT ACC CTT GTT AAT AGA ATC GAG TTA AAA GGT ATT         384
Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly Ile
        115                 120                 125

GAT TTT AAA GAA GAT GGA AAC ATT CTT GGA CAC AAA TTG GAA TAC AAC         432
Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr Asn
    130                 135                 140

TAT AAC TCA CAC AAT GTA TAC ATC ATG GCA GAC AAA CAA AAG AAT GGA         480
Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn Gly
145                 150                 155                 160

ATC AAA GTT AAC TTC AAA ATT AGA CAC AAC ATT GAA GAT GGA AGC GTT         528
Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser Val
                165                 170                 175

CAA CTA GCA GAC CAT TAT CAA CAA AAT ACT CCA ATT GGC GAT GGC CCT         576
Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly Pro
            180                 185                 190

GTC CTT TTA CCA GAC AAC CAT TAC CTG TCC ACA CAA TCT GCC CTT TCG         624
Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu Ser
        195                 200                 205

AAA GAT CCC AAC GAA AAG AGA GAC CAC ATG GTC CTT CTT GAG TTT GTA         672
Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe Val
    210                 215                 220

ACA GCT GCT GGG ATT ACA CAT GGC ATG GAT GAA CTA TAC AAA TA              716
Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Lys
225                 230                 235
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 238 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: protein

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu Val
  1               5                  10                  15

Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly Glu
```

-continued

			20					25					30		
Gly	Glu	Gly	Asp	Ala	Thr	Tyr	Gly	Lys	Leu	Thr	Leu	Lys	Phe	Ile	Cys
		35					40					45			
Thr	Thr	Gly	Lys	Leu	Pro	Val	Pro	Trp	Pro	Thr	Leu	Val	Thr	Thr	Phe
	50					55					60				
Ser	Tyr	Gly	Val	Gln	Cys	Phe	Ser	Arg	Tyr	Pro	Asp	His	Met	Lys	Gln
65					70					75					80
His	Asp	Phe	Phe	Lys	Ser	Ala	Met	Pro	Glu	Gly	Tyr	Val	Gln	Glu	Arg
				85					90					95	
Thr	Ile	Phe	Phe	Lys	Asp	Asp	Gly	Asn	Tyr	Lys	Thr	Arg	Ala	Glu	Val
			100					105					110		
Lys	Phe	Glu	Gly	Asp	Thr	Leu	Val	Asn	Arg	Ile	Glu	Leu	Lys	Gly	Ile
		115					120					125			
Asp	Phe	Lys	Glu	Asp	Gly	Asn	Ile	Leu	Gly	His	Lys	Leu	Glu	Tyr	Asn
	130					135					140				
Tyr	Asn	Ser	His	Asn	Val	Tyr	Ile	Met	Ala	Asp	Lys	Gln	Lys	Asn	Gly
145					150					155					160
Ile	Lys	Val	Asn	Phe	Lys	Ile	Arg	His	Asn	Ile	Glu	Asp	Gly	Ser	Val
				165					170					175	
Gln	Leu	Ala	Asp	His	Tyr	Gln	Gln	Asn	Thr	Pro	Ile	Gly	Asp	Gly	Pro
			180					185					190		
Val	Leu	Leu	Pro	Asp	Asn	His	Tyr	Leu	Ser	Thr	Gln	Ser	Ala	Leu	Ser
		195					200					205			
Lys	Asp	Pro	Asn	Glu	Lys	Arg	Asp	His	Met	Val	Leu	Leu	Glu	Phe	Val
	210					215					220				
Thr	Ala	Ala	Gly	Ile	Thr	His	Gly	Met	Asp	Glu	Leu	Tyr	Lys		
225					230					235					

What is claimed is:

1. A composition of matters comprising:

a fluorescent modified form of an Aequorea wild-type GPP polypeptide,

characterized in that upon oxidation and cyclization of amino acid residues in said fluorescent modified form corresponding to positions 65 to 67 of wild-type GFP polypeptide sequence (SEQ ID NO:2) said fluorescent modified form exhibits a different excitation and/or emission spectrum from a corresponding product of said wild-type GFP polypeptide sequence,

with the proviso that when said fluorescent modified form comprises a mutation at S65, said mutation at S65 is selected from the group consisting of S65A, S65C, S65T, S65L, S65V, and S65I.

2. The composition according to claim 1,

wherein said fluorescent modified form exhibits and alteration in the ration of two main excitation peaks relative to said wild-type GFP polypeptide sequence.

3. The composition according to claim 2,

wherein said fluorescent modified form exhibits increased fluorescence at a shorter-wavelength peak of the two main excitation peaks than said wild-type GFP polypeptide sequence.

4. The composition according to claim 3,

wherein said fluorescent modified form comprises a replacement of Ser at a position corresponding to position 202 in said wild-type GFP polypeptide sequence by Phe and a replacement of Thr at a position corresponding to position 203 by Ile.

5. The composition according to claim 2,

wherein said fluorescent modified form exhibits increased fluorescence at a longer-wavelength peak of the two main excitation peaks than said wild-type GFP polypeptide sequence.

6. The composition according to claim 5,

wherein said fluorescent modified form comprises a replacement of Ile at a position corresponding to position 167 of said wild-type GFP polypeptide sequence by Val or Thr.

7. The composition according to claim 5,

wherein said fluorescent modified form comprises a replacement of Ser at a position corresponding to position 65 of said wild-type GFP sequence by Thr, a replacement of Met at position 153 with Ala, and a replacement of Lys at position 238 with Glu.

8. The composition according to claim 1,

wherein said fluorescent modified form fluoresces at a shorter wavelength than said wild-type GFP polypeptide sequence.

9. The composition according to claim 8,

wherein said fluorescent modified form comprises a replacement of Tyr at a position corresponding to position 66 of said wild-type GFP polypeptide sequence by Phe, His or Trp.

10. The composition according to claim 8,

wherein said fluorescent modified form comprises a replacement of Tyr at a position corresponding to position 66 of said wild-type GFP polypeptide sequence by His and a replacement of Tyr at position 145 with Phe.

11. The composition according to claim 8,
wherein said fluorescent modified form comprises a replacement of Tyr at a position corresponding to position 66 of said wild-type GFP polypeptide sequence by Trp, a replacement of Asn at position 146 by Ile, a replacement of Met at position 153 by Thr, a replacement of Val at position 163 by Ala, and a replacement of Asn at position 212 by Lys.

12. The composition according to claim 8,
wherein said fluorescent modified form comprises a replacement of Tyr at a position corresponding to position 66 of said wild-type GFP polypeptide sequence by Trp, a replacement of Ile at position 123 by Val, a replacement of Tyr at position 145 by His, a replacement of His at position 148 by Arg, a replacement of Met at position 153 by Thr, a replacement of Val at position 163 by Ala, and a replacement of Asn at position 212 by Lys.

13. The composition according to claim 1,
wherein said fluorescent modified form exhibits enhanced emission relative to said wild-type GFP polypeptide sequence.

14. The composition according to claim 13,
wherein said fluorescent modified comprises a replacement of Ser at a position corresponding to position 65 of said wild-type GFP polypeptide sequence by an amino acid selected from the group consisting of Ala, Cys, Thr, Leu, Val and Ile.

15. The composition according to claim 14,
wherein said amino acid is Cys of Thr.

16. A functional mutant fluorescent protein, comprising:
a protein with an amino acid sequence that differs from an amino acid sequence of an Aequorea wild type green fluorescent protein (SEQ ID NO:2) by at least one amino acid substitution that is at position 65, wherein said at least one substitution is either S65A, S65C, S65T, S65L, S65V, or S65I,
wherein said functional mutant fluorescent protein has an excitation or emission different from an excitation spectrum or emission spectrum of said Aequorea wild type green fluorescent protein.

17. The functional mutant fluorescent protein of claim 16,
wherein said at least one amino acid substitution that is at position 65 is S65A.

18. The functional mutant fluorescent protein of claim 17,
wherein said functional mutant fluorescent protein comprises a fusion protein.

19. The functional mutant fluorescent protein of claim 16,
wherein said at least one amino acid substitution that is at position 65 is S65C.

20. The functional mutant fluorescent protein of claim 19,
wherein said functional mutant fluorescent protein comprises a fusion protein.

21. The functional mutant fluorescent protein of claim 16,
wherein said at least one amino acid substitution that is at position 65 is S65T.

22. The functional mutant fluorescent protein of claim 21,
wherein said functional mutant fluorescent protein comprises a fusion protein.

23. The functional mutant fluorescent protein of claim 16,
wherein said at least one amino acid substitution that is at position 65 is S65L.

24. The functional mutant fluorescent protein of claim 23,
wherein said functional mutant fluorescent protein comprises a fusion protein.

25. The functional mutant fluorescent protein of claim 16,
wherein said at least one amino acid substitution that is at position 65 is S65V.

26. The functional mutant fluorescent protein of claim 25,
wherein said functional mutant fluorescent protein comprises a fusion protein.

27. The functional mutant fluorescent protein of claim 16,
wherein said at least one amino acid substitution that is at position 65 is S65I.

28. The functional mutant fluorescent protein of claim 27,
wherein said functional mutant fluorescent protein comprises a fusion protein.

29. The functional mutant fluorescent protein of claim 16,
wherein said functional mutant fluorescent protein consists of mutations S65T, M153A, and K238E.

30. The functional mutant fluorescent protein of claim 29,
wherein said functional mutant fluorescent protein comprises a fusion protein.

31. The functional mutant fluorescent protein of claim 16,
wherein said functional mutant fluorescent protein further comprises an amino acid sequence which targets said protein to the specific cellular locations.

32. A functional mutant fluorescent protein, comprising:
a protein with an amino acid sequence that differs from an amino acid sequence of an Aequorea wild type green fluorescent protein (SEQ ID NO:2) by at least one amino acid substitution that is at position 66, wherein said at least one substitution is either Y66H or Y66W, further wherein said functional mutant fluorescent protein has an excitation or emission different from an excitation spectrum or emission spectrum of said Aequorea wild type green fluorescent protein.

33. The functional mutant fluorescent protein of claim 32,
wherein said at least one amino acid substitution that is at position 66 is Y66W.

34. The functional mutant fluorescent protein of claim 33,
wherein said functional mutant fluorescent protein comprises a fusion protein.

35. The functional mutant fluorescent protein of claim 32,
wherein said at least one amino acid substitution that is at position 66 is Y66H.

36. The functional mutant fluorescent protein of claim 35,
wherein said functional mutant fluorescent protein comprises a fusion protein.

37. The functional mutant fluorescent protein of claim 32,
wherein said functional mutant fluorescent protein consists of mutations Y66H and Y145F.

38. The functional mutant fluorescent protein of claim 37,
wherein said functional mutant fluorescent protein comprises a fusion protein.

39. The functional mutant fluorescent protein of claim 32,
wherein said functional mutant fluorescent protein consists of mutations Y66W, N146I, M153T, V163A and N212K.

40. The functional mutant fluorescent protein of claim 39,
wherein said functional mutant fluorescent protein comprises a fusion protein.

41. The functional mutant fluorescent protein of claim 32,
wherein said functional mutant fluorescent protein consists of mutations Y66W, I123V, Y145H, H148R, M153T, V163A, and N212K.

42. The functional mutant fluorescent protein of claim 41,
wherein said functional mutant fluorescent protein comprises a fusion protein.

43. A functional mutant fluorescent protein, comprising:

a protein with an amino acid sequence that differs from an amino acid sequence of an Aequorea wild type green fluorescent protein (SEQ ID NO:2) by at least one amino acid substitution in the region consisting of positions 65 and 66.

wherein said functional mutant fluorescent protein has an excitation or emission different from an excitation spectrum or emission spectrum of said Aequorea wild type green fluorescent protein.

44. The functional mutant fluorescent protein of claim 43.

wherein said functional mutant fluorescent protein exhibits an alteration in the ratio of two main excitation peaks relative to Aequorea wild type green fluorescent protein.

45. The functional mutant fluorescent protein of claim 44.

wherein said functional mutant fluorescent protein exhibits increased fluorescence at the shorter-wavelength peak of said two main excitation peaks.

46. The functional mutant fluorescent protein of claim 43.

further comprising an amino acid substitution that is at position 202.

47. The functional mutant fluorescent protein of claim 46.

wherein said amino acid substitution that is at position 202 is S202F.

48. The functional mutant fluorescent protein of claim 43.

further comprising an amino acid substitution that is at position 203.

49. The functional mutant fluorescent protein of claim 48.

wherein said amino acid substitution that is at position 203 is T203L

50. The functional mutant fluorescent protein of claim 43.

further comprising an amino acid substitution that is at position 167.

51. The functional mutant fluorescent protein of claim 50.

wherein said amino acid substitution that is at position 167 is I167 V or I167T.

52. The functional mutant fluorescent protein of claim 43.

further comprising an amino acid substitution that is at position 153.

53. The functional mutant fluorescent protein of claim 52.

wherein said amino acid substitution that is at position 153 is M153T or M153A.

54. The functional mutant fluorescent protein of claim 43.

further comprising an amino acid substitution that is at position 238.

55. The functional mutant fluorescent protein of claim 54.

wherein said amino acid substitution that is at position 238 is K238E.

56. The functional mutant fluorescent protein of claim 43.

further comprising an amino acid substitution that is at position 145.

57. The functional mutant fluorescent protein of claim 56.

wherein said amino acid substitution that is at position 145 is Y145H or Y145F.

58. The functional mutant fluorescent protein of claim 43.

further comprising an amino acid substitution that is at position 146.

59. The functional mutant fluorescent protein of claim 58.

wherein said amino acid substitution that is at position 146 is N146I.

60. The functional mutant fluorescent protein of claim 43.

further comprising an amino acid substitution that is at position 163 or 148.

61. The functional mutant fluorescent protein of claim 60.

wherein said amino acid substitution that is at position 163 is V163A and said amino acid substitution at position 148 is H148R.

62. The functional fluorescent protein of claim 43.

further comprising an amino acid substitution that is at position 212 or 123.

63. The functional fluorescent protein of claim 62.

wherein said amino acid substitution that is at position 212 is N212K and said amino acid substitution at position 123 is I123V.

64. The functional fluorescent protein of claim 43.

wherein said functional fluorescent protein comprises a fusion protein.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT : **5,777,079**

DATED : **July 7, 1998**

INVENTOR(S) : **Roger Y. Tsien, et al.**

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

On the title page, under "OTHER PUBLICATIONS", add the following references:

Baldwin et al., Biochemistry 29:5509-9915 (1990)

Chalfie et al., Science 263:802-805 (1994)

Cody et al., Biochemistry 32:1212-1218 (1993)

Cubitt et al., Trends in Biochem. Sci. 20:488-455 (1995)

Delagrave et al., Bio/Technology 13:151-154 (1995)

Deschamps et al., Protein Expression and Purification, 6:555-558 (1995)

Ehrig et al., FEBS Letters 367:163-166 (1995)

Heim et al., Proc. Natl. Acad. Sci. U.S.A. 91:12501-12505 (1994)

Heim et al. Nature 373:663-664 (1995)

Kain et al, BioTechniques 19:650-655 (1995)

Levine et al., Comp. Biochem. Physiol. 728:77-85 (1982).

Muhlrad et al., Yeast 8:79-82 (1992)

Norris et al., Plant Molecular Biology, 24:673-677 (1994)

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT : **5,777,079**

DATED : **July 7, 1998**

INVENTOR(S) : **Roger Y. Tsien, et al.**

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Prasher et al., Gene 111:229-233 (1992)

Sala-Newby et al, Biochem. J. 279:727-732 (1991)

Surpin et al., Photochem. Photobiol. 49:Abstract, 25S (1989)

Ward, in Bioluminescence and Chemiluminescence (eds. DeLuca et al., 235-242 (Academic Press, New York, 1981)

Ward et al., Biochemistry 21:4535-4540 (1982)

Ward et al., Photochem. Photobiol. 35:803-808 (1982)

Wilbanks et al., J. Biol. Chem. 268:1226-1235 (1993)

Signed and Sealed this

Second Day of March, 1999

Attest:

Attesting Officer

Q. TODD DICKINSON

Acting Commissioner of Patents and Trademarks

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,777,079

DATED : 7/7/98

INVENTOR(S) : Tsien et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In column 15, line 40, of claim 1, replace "GPP" with --GFP--.
In column 15, line 55, of claim 2, replace "ration" with --ratio--.
In column 17, line 31, of claim 15, replace "of" with --or--.
In column 17, line 38, of claim 16, replace "S651" with --S65I--.

Signed and Sealed this

Eighth Day of June, 1999

Attest:

Q. TODD DICKINSON

Attesting Officer

Acting Commissioner of Patents and Trademarks

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,777,079

DATED : 7/7/98

INVENTOR(S) : Tsien et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Columns 13-16:

In SEQ ID NO:1, nucleic acid base number 239, replace the "A" with --G--.
In SEQ ID NO:1, amino acid number 80, replace the "Gln" with --Arg--.
In SEQ ID NO:2, amino acid number 80, replace the "Gln" with --Arg--.

Signed and Sealed this

Thirty-first Day of August, 1999

Attest:

Q. TODD DICKINSON

Attesting Officer

Acting Commissioner of Patents and Trademarks